AD_____

Award Number: DAMD17-00-1-0665

TITLE: Amplification of DNA Topoisomerase II and c-erbB2 in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Joseph A. Holden, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Utah
Salt Lake City, Utah 84102

REPORT DATE: September 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2001	Final (1 Sep 00 - 31 Aug 01)

4. TITLE AND SUBTITLE
Amplification of DNA Topoisomerase II and c-erbB2 in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0665

6. AUTHOR(S)
Joseph A. Holden, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Utah
Salt Lake City, Utah 84102

E-Mail: **joe.holden@path.med.utah.edu**

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Elevations of the c-erbB2 oncogene occur in about 30% of human breast cancers. Patients whose tumors show elevation of the c-erbB2 oncogene respond to anti-cancer drugs targeted against DNA topoisomerase II (topo II). The gene for topo II is closely linked to the gene for c-erbB2 on chromosome 17. Laboratory data suggest that co-amplification of topo II with c-erbB2 occurs and renders tumor cells sensitive to drugs targeting topo II. This suggests that the reason patients with breast cancers showing amplification of c-erbB2 respond to topo II anticancer drugs is because of the co-amplification of topo II. This study used a novel quantitative polymerase chain reaction (PCR) method to determine the frequency of topo II and c-erbB2 gene amplification in samples of human breast cancer. Of 31 cases of breast cancer which showed c-erbB2 gene amplification by PCR, only 3 showed co-amplification of topo II. Therefore, co-amplification of both genes in human breast cancer is not a common event and probably does not explain the sensitivity of such tumors to topo II drug therapy. Alternatively, we obsevered high topo II protein expression in c-erbB2 amplified tumors. This most likely reflects the high number of proliferating cells in these tumors and may be the explanation for the sensitivity to topo II drugs.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, topisomerase II, c-erbB2, polymerase chain reaction	67
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Elevations of the c-erbB2oncogene occur in about 30% of human breast cancers. Interestingly, patients whose tumors show elevation of the c-erbB2 oncogene are sensitive to chemotherapeutic protocols involving drugs targeted against DNA topoisomerase II-alpha (topo II) (1). The gene for c-erbB2 is on chromosome 17 and is linked to the gene for topo II. Both genes have been found to be amplified together in breast cancer cells grown in the laboratory (2). These cancer cells with amplification of both genes are exquisitely sensitive to drugs which target topoII. This leads to the intriguing hypothesis that the reason patients with amplification of c-erbB2 are sensitive to topo II anticancer drugs is because of the co-amplification of c-erbB2 with topo II. The purpose of this project was to determine how often topo II and c-erbB2 are co-amplified in cases of human breast cancer.

Body

To determine how often the topo II gene is co-amplified with the c-erbB2 oncogene, we developed a quantitative polymerase chain reaction (PCR) assay to determine the gene copy number of topo II and c-erbB2 relative to albumin in samples of human breast cancer. Tumors with elevated c-erbB2 were identified by immunohistochemical staining. Tissue from these tumors was micro-dissected from an unstained slide, the DNA was isolated by standard procedures, and then the amplification of topo II with c-erbB2 was determined by PCR with appropriate primers. Of the 31 cases which we found amplified for c-erbB2, only 3 cases showed amplification of topo II. A detailed description of the methods, results, and conclusions has been submitted for publication (3)and a copy of the submitted manuscript is included in the Appendix.

Key Research Accomplishments

1. We developed a novel method to determine gene copy number from micro-dissected tumor tissue.
2. We were able to correlate topo II and c-erbB2 gene amplification with immunohistochemical results.
3. We observed that many samples interpreted as c-erbB2 amplified by immunostaining (2+ staining) do not show c-erbB2 amplification by PCR or fluorescence in situ hybridization (FISH).
4. We discovered that the co-amplification of the topo II gene with the c-erbB2 oncogene is not a common event in human breast cancer.

Reportable Outcomes

1. Bernard PS, et al., Color multiplex PCR for the quantitative analysis of predictive genes in breast cancer: c-erbB2 and topoisomerase II-alpha. (submitted for publication)
2. Bernard PS, et al., HER2/neu and topoisomerase II-alpha Linked genes with prognostic and predictive value in invasive ductal cell breast carcinoma. The Eleventh Conference on DNA Topoisomerases in Therapy, NYU Medical School, New York, October, 2001

Conclusions

The question we sought to answer with this study was whether the topo II gene is co-amplified with c-erbB2 in cases of human breast cancer. Such a co-amplification might then explain the known sensitivity of c-erbB2 amplified tumors to topo II targeted anticancer drugs. Of 31 human breast cancers found to have elevated c-erbB2 by PCR, only 7 showed alterations in the topo II gene. Of these 7 tumors with topo gene alterations, only 3 showed gene amplification. We conclude that co-amplification of topo II and c-erbB2 is a rare event in human breast cancer and can not explain the sensitivity of such tumors to drugs targeting topo II. However, we observed, by immunohistochemical staining, elevated expression of topo II protein in c-erbB2 amplified tumors. Topo II is a marker of cell proliferation (4). The abundant topo II expression in c-erbB2 amplified tumors most likely reflects that these breast cancers are composed of a large fraction of proliferating cells. Thus our data suggest that the reason c-erbB2 amplified breast cancers are sensitive to topo II targeting drugs is not because of co-amplification of the gene with c-erbB2, but rather because these tumors have a high expression of the protein. This conclusion has broad implications beyond breast cancer because it suggests that human cancers responsive to drugs targeting topo II could be identified by immunohistochemical staining for the protein.

References

1. Muss HB., et al., c-erbB2 expression and response to adjuvant therapy in women with node-positive early breast cancer. New England J Medicine, 330:1260-1266, 1994

2. Coutts J., et al., Expression of topoisomerase II-alpha and beta in an adenocarcinoma cell line carryingamplified topoisomerase II-alpha and retinoic acid receptor alpha genes. British J Cancer, 68:793-800, 1993

3. Bernard PS., et al., Color multiplex PCR for the quantitative analysis of predictive genes in breast cancer: c-erbB2 and topoisomerase II-alpha (submitted for publication) 2001

4. Lynch BS., et al., Human DNA topoisomerase II-alpha: a new marker of cell proliferation in invasive breast cancer. Human Pathology, 28:1180-1188, 1997

Appendix

1. Bernard PS., et al., Color multiplex PCR for the quantitative analysis of predictive genes in breast cancer: c-erbB2 and topoisomerase II-alpha (submitted for publication)

2. HOlden, JA. DNA topoisomerases as anticancer drug targets: from the laboratory to the clinic. Current Medicinal Chemistry-Anti-Cancer Agents, 1:1-15, 2001

Color Multiplex PCR for the Quantitative Analysis of Predictive Genes in Breast Cancer: c-*erb*B-2 and topoisomerase IIα[1]

Philip S. Bernard, Carl T. Wittwer, Randy P. Rasmussen, David J. Eyre, Mathew A. Scullion, Jason M. Schallheim, Lester J. Layfield, and Joseph A. Holden[2]

Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah 84132 [P.S.B., C.T.W., L.J.L., J.A.H.];
Idaho Technology Inc., 390 Wakara Way, Salt Lake City 84108 [R.P.R., D.J.E, M.A.S., J.M.S.]

Running Title: Quantitative Analysis of c-*erb*B-2 and topo IIα

Key Words: c-*erb*B-2, topoisomerase IIα, multiplex, PCR, breast

[1] Supported in part by an STTR Grant (GM-58983) from the NIH to C.T.W. and a Grant from the Department of Defense (DAMD170010665) to J.A.H. Presented at the United States and Canadian Academy of Pathology, March 3-9, 2001, Atlanta, GA.
[2] To whom requests for reprints should be addressed at University of Utah School of Medicine, Department of Pathology, 50 North Medical Drive, SLC, UT Phone: (801) 581-4958 Fax: (801) 585-3831 E-mail: joe.holden@path.utah.edu.

ABSTRACT

The use of molecular markers in breast cancer is important for prognosis and selecting proper treatment. The presence of amplification or overexpression of the c-*erb*B-2 gene is a negative prognostic factor that is proving to be predictive for response to monoclonal therapy with trastuzumab and first line anthracycline therapy. While response to trastuzumab is effective by blocking c-*erb*B-2 signaling and immune mediated cytotoxicity, the reason why anthracyclines are effective in these tumors is less clear. The c-*erb*B-2 gene is physically and biologically associated with topoisomerase IIα, the target of anthracyclines. In tumors with c-*erb*B-2 gene amplification there can be concomitant alterations in topoisomerase IIα, which may confer resistance (deletion) or sensitivity (amplification) to anthracyclines such as doxorubicin. We scored for the c-*erb*B-2 and topoisomerase IIα markers in a group of 59 ductal cell breast carcinomas (53 invasive and 6 *in-situ*) using immunohistochemistry (IHC), fluorescence in-situ hybridization (FISH, c-*erb*B-2 only), and real-time quantitative PCR. All samples were initially selected based on a positive IHC score for c-*erb*B-2. Immunohistochemistry was performed for topoisomerase IIα to assess proliferation by the topo II index. Seven out of 31 (23%) of the samples found amplified for the c-*erb*B-2 gene by PCR had either a deletion (4/31) or amplification (3/31) in the topoisomerase IIα gene. Using the CB11 clone for c-*erb*B-2, there was 90% agreement between PCR and FISH when IHC was strongly positive (i.e., scored 3^{+}). The concordance between methods was less when the A0485 polyclonal antibody was used and for tumors that scored weakly positive. The low frequency of concomitant DNA alterations between c-*erb*B-2 and topoisomerase IIα suggest that the response of c-*erb*B-2 positive breast cancers to anthracyclines is not

dependent on co-amplification of the topoisomerase IIα gene. The color multiplex PCR method has general diagnostic application for the rapid, simultaneous, and quantitative analysis of many targets from microdissected tissue.

Introduction

The c-*erb*B-2[3] proto-oncogene encodes a 185-kDa transmembrane glycoprotein that heterodimerizes with other members of the epidermal growth factor receptor family resulting in tyrosine kinase activation, autophosphorylation and cell transformation (1). In approximately 20% of breast cancers, the c-*erb*B-2 gene becomes amplified at the DNA level leading to an increase in message and overexpression of the protein (2,3). Amplification and overexpression of c-*erb*B-2 are associated with aggressive breast tumor transformation and poor clinical outcome in women with node-positive and node-negative breast cancer (4-7). In addition to its prognostic relevance, clinical trials are evaluating the predictive importance of c-*erb*B-2 (8). Current data suggest that the presence of this marker is a strong positive predictive indicator of response using first-line chemotherapeutics (e.g., anthracyclines) in combination with trastuzamab (8-10).

Trastuzumab (Herceptin) is a recombinant humanized monoclonal antibody (humAb4D5) targeting the extracellular domain of c-*erb*B-2 (11). The drug inhibits the growth of breast cancer cells overexpressing c-*erb*B-2 in culture and in human xenografts (11-14). Monoclonal antibodies against c-*erb*B-2 are effective inhibitors of breast cancer progression by antagonizing mitogenic signal transduction and through antibody-dependent cell-mediated cytotoxicity (15,16). While it is biologically rational to expect

[3] also known as HER-2/*neu*

trastuzumab to be effective therapy against c-*erb*B-2 positive tumors (16), the reason why anthracyclines also work against this class of tumors is less obvious (8).

It is suspected that anthracyclines, such as doxorubicin, are effective against c-*erb*B-2 positive breast tumors because of an association with topo IIα[4] (17-20). The topo IIα gene is physically located near c-*erb*B-2 within the chromosome band region 17q12-q21, an area that is frequently modified in breast tumors (21-23). If c-*erb*B-2 is amplified the topo IIα gene may also be amplified or deleted resulting in corresponding changes in protein expression (19). The topo IIα protein is a dimeric enzyme (170 kDa subunits) necessary for DNA breakage and strand passage during cell proliferation (24). The general class of drugs known as topo II inhibitors, which include anthracyclines and other topo II poisons, target the topo IIα protein resulting in unrepaired DNA breaks during cell division and cell death by apoptosis (25-27). In vitro studies have shown that gene deletion of topo IIα confers drug resistance to doxorubicin by downregulating protein expression (19,28). Conversely, cell lines overexpressing topo IIα, either through transfection (29,30) or gene amplification (19), are sensitive to topo II inhibitors.

Since topo IIα is cyclically expressed primarily in the late S/G2 phases of the cell cycle, it is a reliable marker of cell proliferation by IHC (31,32). Immunochemistry for topo IIα is a semi-quantitative technique, which is not optimal for assessing small differences in expression (27). This may partly explain why topo IIα as a proliferation marker was found in one study not to be predictive for response to anthracyclines in patients (33). Recently, FISH and quantitative PCR have found that amplification or deletion of topo IIα is a common event in c-*erb*B-2 positive breast tumors (23,34). Given

[4] abbreviation used: topo IIα, topoisomerase IIα

the strong in vitro evidence (19,28-30), Jarvinen et al contend that these alterations could be predictive and may significantly account for variability in response to topo II inhibitors in patients (19).

We used color multiplex real-time quantitative PCR to accurately determine alterations in c-*erb*B-2 and topo IIα, relative to a control gene. Our data suggest that gene alterations in topo IIα are infrequent in breast cancer and could account for only a small percentage (<5%) of the variability in response to adjuvant topo II inhibitor therapy seen in women with c-*erb*B-2 positive tumors. Moreover, proliferation by the topo II index remains clinically useful by indicating the aggressiveness of a tumor and the presence of target for topo II inhibitor drugs, regardless of c-*erb*B-2 status.

Materials and Methods

Sample Collection. Fifty-three invasive and 6 *in-situ* ductal cell breast carcinomas, formalin-fixed and paraffin-embedded, were received in the Department of Surgical Pathology at The University of Utah School of Medicine (SLC, UT). All identifiers were removed from samples to protect patient confidentiality. Samples were pre-selected based on positive c-*erb*B-2 status using IHC. Scoring criteria were according to the FDA approved HercepTest™. Specifically, those samples scored 2^+ (weak, continuous membrane staining in 10% of tumor cells) and 3^+ (strong, continuous membrane staining in at least 10% of tumor cells) were selected for further study by FISH and PCR.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue was cut onto charged glass slides in 5-μm thick sections. For c-*erb*B-2 staining, sections were deparaffinized and subjected to heat-induced epitope retrieval (HIER) by immersion in 10 mM citrate buffer (pH 6.0) in a microwave (half-power) for 15 min. Samples were cooled to room temperature in buffer for an additional 15 min. Primary antibody dilutions for CB11 and the Dako polyclonal (A0485) were prepared according to manufacturer's instructions. Samples were incubated with antibody using the Ventana 320 immunostainer (Ventana Medical Systems, Tucson, AZ) and color development was accomplished with diaminobenzidine. Topo IIα protein was stained by IHC using a similar method described elsewhere (32). Proliferation was determined using the topo II index, which is defined as the number of positive-staining cells divided by the total number of tumor cells counted times 100. At least 500 tumor cells were counted in the area of highest staining. Immunohistochemistry was performed and interpreted on all samples within 1 week of the tissue being paraffin-embedded.

Fluorescence *in-situ* Hybridization. To determine c-*erb*B-2 status by FISH, a commercial kit was obtained and used following manufacturer's instructions (Oncor® INFORM™ HER-2/neu Gene Detection System, Ventana Medical Systems, Tucson, AZ). Briefly, formalin-fixed, paraffin-embedded sections were dried overnight at 65 °C, then deparaffinized in xylene and washed in ethanol. Samples were subjected to enzymatic protein digestion for 40 min at 37 °C, denatured at 75 °C and then incubated overnight with c-*erb*B-2 specific probe under a cover slip. After a post-hybridization washing,

detection was performed using indirect labeling of fluorescein tagged avidin. Specimens were evaluated under oil immersion at X100 magnification using an epifluorescence microscope equipped with the appropriate filters. Samples with a mean fluorescence signal count greater than 4, after analyzing 20 cells, were considered amplified.

Microdissection and DNA Extraction. Samples were laser-capture microdissected and DNA was extracted according to standard protocols (36). Thin-sections of paraffin-embedded tissue were cut onto untreated slides, deparaffinized, and lightly stained with hematoxylin (30 sec) and eosin (5 sec). Each H&E stained sample was aligned with a consecutive section that was IHC stained for c-*erb*B-2. The c-*erb*B-2 positive area was identified on the H&E slide and circled by a pathologist with an indelible fine felt tip marker. For laser-capture microdissection, approximately 500 cells within the circled area were collected onto a CapSure transfer film using the PixCell® instrument (Arcturus Engineering Inc., Mountain View, CA, USA). Alternatively, if the circled area contained confluent tumor without lymphocyte infiltration, the area was scalpel dissected with the aid of light microscopy. The DNA from the microdissected tissue was extracted overnight at 37 °C in 100 µl of digestion buffer containing 1 mg/ml Proteinase K and 1% Tween 20 in Tris-EDTA, pH 8.0. Samples were centrifuged (5 min @ 8,000 rpm), Proteinase K heat inactivated (95 °C for 8 min) and DNA purified using DNeasy™ Tissue Kit (Qiagen Inc., USA) with a poly[dA] carrier (Sigma®, USA). The DNA from each sample was ethanol precipitated in the presence of sodium chloride. The pellet was frozen at - 20 °C until PCR and then resuspended in 6 µl of 10 mM Tris, 0.1 mM EDTA, pH 8.0.

Instrumentation and Dyes for 3-Color Multiplexing. A linear optics block, similar to that in the LightCycler™ (37), was equipped with the appropriate filters to perform 3-color multiplexing using the dyes LCRed640, Cy5 and LCRed705. All 3 acceptor dyes used fluorescein as a common donor. Although each acceptor has a discrete emission maximum, there is some overlap that is corrected by software. The software algorithm used for color compensation of spectral overlap between fluorescent dyes models that used in flow cytometry with modifications for solution chemistry and temperature dependence of fluorescence (38). The new optics block was installed into a R.A.P.I.D. (Ruggedized Advanced Pathogen Identification Device) – a durable version of the LightCycler™ commercially marketed to the military by Idaho Technology Inc. (Salt Lake City, UT)

Primers, Probes and PCR. Oligonucleotides were synthesized by standard phosphoramidite chemistry. Primers were desalted and fluorescent probes were HPLC purified by the manufacturer (IT Biochem, Idaho Technology Inc.). Primer and probe sequences are provided in Table 1. The T_ms of the primers and probes were matched between targets. Probe T_ms were designed to be approximately 5 °C above those of the primers to ensure hybridization during fluorescence acquisition. All amplicons were 100-150 bps in length. Initial selection of primers and probes was done using Primer Designer© 4 (Scientific and Educational Software, State Line, PA) and the T_ms were recalculated for the concentration of $MgCl_2$ used in the PCR[5]. All targets were amplified together and fluorescence acquired once per cycle through 45 cycles (94 °C, 0 s; 64 °C, 10 s). The probe sets for each target were present at the following concentrations: c-*erb*B-

[5] Available under TM Utility at http://www.Idahotech.com.

2 - 0.2 μM fluorescein-labeled probe, 0.2 μM LCRed640-labeled probe; albumin - 0.2 μM fluorescein-labeled probe, 0.2 μM Cy5-labeled probe labeled probe; and topoisomerase IIα - 0.4 μM fluorescein-labeled probe, 0.4 μM LCRed705-labeled probe. In addition, each 10 μl reaction contained 3 mM $MgCl_2$, 50 mM Tris, pH 8.3 (25 °C), 500 μg/ml bovine serum albumin, 0.2 mM each dinucleoside triphosphate, 0.5 μM each primer, 1 U Klentaq1™, and 0.2 μg TaqStart antibody (CLONTECH Laboratories, Palo Alto, CA).

Efficiency Adjustment. Efficiency curves were established within each run using 4 PCR reaction cuvettes containing 10-fold differences in concentration (100 ng to 0.1 ng) of serially diluted human leukocyte genomic DNA (a renewable source of wild-type DNA). Each cuvette contained a multiplex reaction with primers and probes for simultaneously amplifying c-*erb*B-2, topo IIα, and albumin. After color compensation, amplification curves for each target were constructed by plotting cycle number versus fluorescence signal. The second derivative maximum of the amplification curve is denoted the crossing point (C_p). A standard curve was constructed for each target by plotting C_p versus log ng genomic DNA and finding the least squares linear regression (trend line) through the points. Amplification efficiency was found from the slope of the trend line:

Efficiency = 10^(-1/slope)

The slope of the trend line is a function of the average efficiency of the PCR reaction. The y-intercept of the trend line is a function of the minimum detectable amplicon (39). In order to directly compare a crossing point of one target with a crossing point of another target all crossing points were normalized to match the slope and intercept of c-*erb*B-2 (this was an arbitrary selection since any of the targets could have been used). An example of normalizing the C_p for albumin is provided below:

Normalized C_p = observed C_p + ((*erb*B-2 intercept – alb intercept)/log *erb*B-2 efficiency)

Relative amplification for the genes of interest (i.e., c-*erb*B-2 and topo IIα) was found with respect to the control gene albumin after C_p corrections:

Relative amplification = matched efficiency^($C_{p\ \text{interest gene}} - C_{p\ \text{albumin}}$)

Establishing Normal Cut-Off Values. Amplification and deletion cut-off values for c-*erb*B-2 and topo IIα were determined from 7 different runs and 31 reactions using normal breast tissue genomic DNA. Within run amplification efficiencies were determined as described above. The efficiencies were matched and C_ps corrected for the targets in the test samples (i.e., normal breast). The adjusted C_p for each target in a test sample was used to extrapolate an absolute copy number (y-intercept) from the matched standard curves. Since using a ratio (gene of interest/control gene) results in a non-parametric distribution, copy numbers were normalized to a binomial distribution. For instance, the normalized ratio for c-*erb*B-2 was:

Normalized ratio = copy # c-*erb*B-2/(copy # c-*erb*B-2 + copy # albumin)

A 99% confidence interval (3 standard deviations from the mean) was then used to find cut-off values for c-*erb*B-2 (>2.3, amplified; <0.4, deleted) and topo IIα (>2.3, amplified; <0.5 deleted). These values represent fold amplification or deletion from the control.

Statistical Comparison of PCR to FISH. A contingency table of paired results from PCR and FISH was constructed and tested using the McNemar's test – a type of Chi-squared test (40).

Results

Microdissection and 3-Color Multiplexing. By combining microdissection and quantitative PCR, it was possible to avoid sampling errors and provide an objective method of analysis. Using a prototype thermal cycler with real-time fluorescent capability, we illustrated for the first time that fluorescent hybridization probes allow quantitative analysis for at least 3 different targets within a single reaction. We applied our quantitative PCR assay to simultaneously determine alterations in the DNA copy number of c-*erb*B-2 and topo IIα, relative to the control gene albumin (Figure 1). A serial dilution of wild-type leukocyte DNA was included within each run to generate a standard curve for each target (Fig. 2A). Within run efficiency differences between targets were corrected and matched to a single efficiency curve (Fig. 2B). By analyzing the standard efficiency curves from 20 runs done over a 5-month period, we found the run-to-run variation in efficiency for each of our targets to be: c-*erb*B-2 (E=1.87±0.07), albumin

(E=1.89±0.09), and topo IIα (E=1.87±0.08). Although a linear dynamic range for each target could be shown down to 100 pg (~ 15 cells) of genomic DNA (Figure 2), it was rarely necessary to use such a small sampling. Furthermore, we found the best reproducibility when microdissecting between 500-1,000 cells.

Correlation Between Methods for c-*erb*B-2 Status. Samples initially selected based on a positive c-*erb*B-2 IHC score were further analyzed by FISH and PCR. When considering only those tumors immunostained with the Ventana CB11 monoclonal antibody, the agreement between a strong positive IHC score (i.e., scored 3^+) and an amplified PCR score was 96% (25/26). There were 4 samples stained with the Dako polyclonal (A0485) antibody only. All these samples were scored 3^+ by IHC but were scored as non-amplified by PCR. In contrast to the tumors staining strongly positive for c-*erb*B-2 with the CB11 clone, only 17% (5/29) of the tumors staining weakly positive (i.e., scored 2^+) were found amplified by PCR. The same percentage of samples (4/23) were found amplified for c-*erb*B-2 by FISH. Overall, there was 90% agreement between PCR and FISH for samples scored 3^+ positive by IHC. There was 70% concordance between PCR and FISH for samples scored 2^+ positive by IHC. On average, samples scored c-*erb*B-2 positive by PCR were amplified 3.5-fold (7 copies). By comparison, there was an average of 13 copies per cell in the samples scored c-*erb*B-2 positive by FISH. The same sample was found to have the highest c-*erb*B-2 copy number by both PCR and FISH, determined to be 38 and 20 copies, respectively.

The results from PCR and FISH were compared using the McNemar's test, which is appropriate for determining whether or not 2 methods (in this case PCR and FISH) agree or disagree on their classification of a sample from the same individual (38).

Although data is limited, we could not reject the null hypothesis that PCR is equal to FISH for calling samples positive by IHC (P = 0.74).

Proliferation and c-*erb*B-2 Status. After determining c-*erb*B-2 status by each method, samples were further stratified by proliferation using the topo II index (Table 2). Those tumors c-*erb*B-2 positive by PCR had the highest average topo II proliferation compared to those positive by either IHC or FISH. In addition, as the PCR determined copy number increased for c-*erb*B-2, so did proliferation. This same correlation was not seen for FISH since tumors with greater than 10 copies had on average lower proliferation than those with less than 10 copies. We found no association between the topo II index and either topo IIα amplification or deletion. For instance, 2 of the tumors that were topo IIα deleted had higher a proliferation than most of the non-amplified tumors and even some of the tumors found amplified for topo IIα.

Concomitant Alterations Determined by PCR. Despite the close physical link (~ 1 Mbp) between c-*erb*B-2 and topo IIα, we found that the 2 loci are infrequently co-altered. Thirty-one out of 59 breast carcinomas were c-*erb*B-2 amplified as determined by PCR. Seven (23%) of these 31 samples had a concomitant alteration in the topo IIα gene with approximately an equal number of samples amplified and deleted (Table 3). The 3 samples with concomitant c-*erb*B-2 and topo IIα amplifications had different copy numbers. The copy numbers in these samples were 20, 11, and 9 for c-*erb*B-2 compared to 8, 5 and 11 for topo IIα, respectively. No alterations in topo IIα were found in tumors not amplified for c-*erb*B-2.

Discussion

Advances in genomics and cancer biology are revealing genes that will provide a molecular basis for clinical decision-making regarding appropriate therapy in breast cancer (41,42). Currently, the most accepted tumor markers for use in breast cancer are the estrogen and progesterone hormone receptors (8). When present, these markers give a favorable prognosis and predict response to endocrine therapy. In contrast, the use of c-*erb*B-2 as a predictive marker has been more controversial. Overexpression of c-*erb*B-2 in vitro promotes hormone-independent growth and tamoxifen resistance (43). Human breast cell lines positive for c-*erb*B-2 can be inhibited, however, by the mouse monoclonal antibody 4D5 (11). In addition, randomized clinical trials have shown that trastuzumab, the humanized 4D5 monoclonal therapy, has clinical benefit in women with c-*erb*B-2 positive tumors when used as a single agent or in combination with standard chemotherapies (8,10).

The most effective chemotherapeutic regimens against c-*erb*B-2 positive tumors are anthracycline-based (e.g., those containing doxorubicin). The sensitivity of c-*erb*B-2 positive tumors to doxorubicin is likely related to its effect on changes in regulation of topoisomerase IIα expression or activity (44). In vitro models show that activation of the c-*erb*B-2 pathway leads to overexpression and increased activity of topo IIα resulting in sensitivity to doxorubicin (19,44). Conversely, if trastuzumab is used to block the signaling of c-*erb*B-2, then topo IIα expression and activity are decreased conferring a relative resistance to doxorubicin (44). We found that the c-*erb*B-2 and topo IIα genes, although physically located near each other, are rarely co-altered in human breast tumors.

We contend that topo IIα gene amplification or deletion is not a significant mechanism leading to sensitivity or resistance of human breast cancer to anthracycline based chemotherapy.

Alterations in many other genes could control the transcription and catalytic activity of topo IIα (21,22), which may explain the variability in response of c-*erb*B-2 positive breast tumors to chemotherapy (8,45). For example, the Grb7 gene is frequently co-amplified and overexpressed with c-*erb*B-2, and maps much closer to c-*erb*B-2 than topo IIα (21,42,46). The gene products of c-*erb*B-2 and Grb7 are known to interact directly, and together are likely to up-regulate a signaling pathway in human breast cancer that involves topo IIα. Since many mechanisms yet to be elucidated could be responsible for driving proliferation, methods for detecting the presence of the topo IIα protein alone should be helpful in predicting response to topo II inhibitors. We found that the topo II index correlates with c-*erb*B-2 status. The presence of both topo IIα and c-*erb*B-2 may indicate an intact signaling pathway in which the combination of trastuzumab and anthracyclines will be most effective.

Clinical decision making for the use of trastuzumab in breast cancer should be based on c-*erb*B-2 status. In combination with the recommended anthracycline therapy, this regimen carries a significant risk of cardiotoxicity (8,10). Thus, the additional health risk involved in starting the appropriate therapy for c-*erb*B-2 positive tumors makes having an accurate determination of c-*erb*B-2 status critically important. Unfortunately, there are conflicting results in determining c-*erb*B-2 status depending on the type of assay, reagents and scoring criteria used.

There have been 3 FDA approved diagnostic tests (2 FISH-based and 1 IHC-based) to assess c-*erb*B-2 status (7). While there is excellent agreement between the different FISH assays (98% concordance), there is generally poor agreement between FISH and IHC (47). Particularly, this is found in weakly staining c-*erb*B-2 positive tumors (3,47). Using PCR as another DNA based assay to help resolve discrepancies between IHC and FISH, we found 90% concordance between all 3 methods for tumors scored strongly positive for c-*erb*B-2. In addition, we found that PCR and FISH agree that the majority (>80%) of tumors scored weakly positive for c-*erb*B-2 by IHC are negative for DNA amplification. Although overexpression of c-*erb*B-2 may be due to a mechanism other than gene amplification in these weakly staining samples, Tubbs et al has found that weakly staining (i.e., 2^+) tumors do not overexpress mRNA (3). Finally, it appeared arbitrary to which 2^+ positive IHC samples were called positive by FISH and PCR. That is, PCR and FISH did not agree on which 2^+ IHC samples were gene amplified suggesting that these cases may be false positives for the DNA based methods. Further studies correlating mRNA expression to DNA amplification and IHC should help resolve these remaining discrepancies.

The IHC-based HercepTest was approved to predict response to trastuzumab. Curiously, the CB11 and 4D5 mouse monoclonals, initially used in the preclinical treatment studies and the Herceptin clinical trials, are not the antibodies used in the HercepTest. Instead a goat polyclonal antibody A0485 (Dako) is used. Consistent with other studies (2,48), we found that the A0485 antibody has a different specificity than the CB11 clone and may introduce false positives. The use of antibodies with different protein domain specificities has been an additional source of variability between IHC

methods (49). For example, varying protein expression has been found whether the antibody is specific for the external domain (4D5 or TAB250), the internal domain (CB11), or both (polyclonals).

The major advantages of using PCR over other methods in molecular diagnostics include speed, objectivity, and decreased labor. Although the coupling of microdissection with PCR does not yet provide single cell analysis, the technique greatly improves sensitivity by excluding sample that is not tumor. It is possible that copy number heterogeneity within the tumor itself could account for the lower percentage of concomitant alterations between c-*erb*B-2 and topo IIα found in this study as compared to other work with FISH (19,20). Both PCR and FISH allow DNA from archival tissue to be retrospectively analyzed and correlated to patient outcome without the problem of protein degradation seen with IHC (2,50). Moreover, color multiplex PCR provides internal controls and the ability to analyze several targets of interest without additional sample requirements. We have shown here the simultaneous quantitative analysis of 3 genes from DNA isolated from microdissected samples. As other important targets are identified, it will be necessary to extract a higher density of information from available sample. The need for consecutive tissue sections in FISH and IHC to analyze each target of interest becomes impractical. Color multiplex PCR has been validated in research and will be valuable in clinical cancer medicine for analyzing multiple targets that will guide therapy.

Acknowledgements

We greatly appreciate Sheryl Tripp for her technical assistance and Dr. David Witherspoon for his help with statistical analysis.

REFERENCES

1. Menard, S., Tagliabue, E., Campiglio, M., and Pupa, S.M. Role of HER2 gene overexpression in breast carcinoma. J. Cell. Phys., 182: 150-162, 2000.
2. Pauletti, G., Dandekar, S., Rong, H., Ramos, L., Peng, H., Seshadri, R., and Slamon, D.J. Assessment of methods for tissue-based detection of HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. J. Clin. Oncol., 18: 3651-3664, 2000.
3. Tubbs, R.R., Pettay, J.D., Roche P.C., Stoler, M.H., Jenkins, R.B., and Grogan, T.M. Discrepancies of clinical laboratory testing of eligibility for trastuzumab therapy: apparent immunohistochemical false-positives do not get the message. J. Clin. Oncol., 19: 2714-2721, 2001.
4. Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and McGuire, W.L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science, 235: 177-182, 1987.
5. Paik, S., Hazan, R., Fisher, E.R., Sass, R.E., Fisher, B., Redmond, C., Schlessinger, J., Lippman, M.E., and King, C.R. Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic significance of erbB-2 protein overexpression in primary breast cancer. J. Clin. Oncol., 8: 103-112, 1990.
6. Kallioniemi, O.P., Holli, K., Visakorpi, T., Koivula, T., Helin, H.H., and Isola, J.J. Association of c-erbB-2 protein over-expression with high rate of cell

proliferation, increased risk of visceral metastasis and poor long-term survival in breast cancer. Int. J. Cancer., 49: 650-655, 1991.

7. Ross, J.S., and Fletcher, J.A. The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. Stem Cells, 16: 413-428, 1998.
8. Yamauchi, H., Stearns, V., and Hayes, D.F. When is a tumor marker ready for prime time? A case study of c-erbB-2 as a predictive factor in breast cancer. J. Clin. Oncol., 19: 2334-2356, 2001.
9. Baselga, J. Current and planned clinical trials with trastuzumab (Herceptin). Semin. Oncol., 27 (5 Suppl 9): 27-32, 2000.
10. Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpress HER2. N. Engl. J. Med., 344: 783-792, 2001.
11. Lewis, G.D., Figari, I., Fendly, B., Wong, W.L., Carter, P., Gorman, C., and Shepard, H.M. Differential responses of human tumor cell lines to anti-p185^{HER2} monoclonal antibodies. Cancer Immunol. Immunother., 37: 255-263, 1993.
12. Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B., Henner, D., Wong, W.L., Rowland, A.M., Kotts, C., Carver, M.E., and Shepard, H.M. Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc. Natl. Acad. Sci. USA, 89: 4285-4289, 1992.
13. Hudziak, R.M., Lewis, G.D., Winget, M., Fendly, B.M., Shepard, H.M., and Ullrich, A. p185^{HER2} monoclonal antibody has antiproliferative effects in vitro,

and sensitizes human breast tumor cells to tumor necrosis factor. Mol. Cell. Biol., 9: 1165-1172, 1989.

14. Baselga, J., Norton, L., Albanell, J., Kim, Y-M, and Mendelsohn, J. Recombinant humanized anti-HER2 antibody (Herceptin™) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. Cancer Res., 58: 2825-2831, 1998.
15. Sliwkowski, M.X., Lofgren, J.A., Lewis, G.D., Hotaling, T.E., Fendly, B.M., and Fox, J.A. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). Semin. Oncol., 26 (4 Suppl. 12): 60-70, 1999.
16. Pegram, M., and Slamon D. Biological rationale for HER2/neu (c-erbB2) as a target for monoclonal antibody therapy. Semin. Oncol., 27 (5 Suppl. 9): 13-19, 2000.
17. Jarvinen, T.A., Kononen, J., Pelto-Huikko, M., and Isola, J. Expression of topoisomerase IIalpha is associated with rapid cell proliferation, aneuploidy, and c-erbB2 overexpression in breast cancer. Am. J. Pathol., 148: 2073-2082, 1996.
18. Smith, K., Houlbrook, S., Greenall, M., Carmichael, J., and Harris, A.L. Topoisomerase II alpha co-amplification with erbB2 in human breast cancer and breast cancer cell lines: relationship to AMSA and mitoxantrone sensitivity. Oncogene, 8: 933-938, 1993.
19. Jarvinen, T.A., Tanner, M., Rantanen, V., Barlund, M., Borg, A., Grenman, S., and Isola, J. Amplification and deletion of topoisomerase IIα associate with erbB-2 amplification and affect sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer. Am. J. Pathol., 156: 839-847, 2000.

20. Tanner, M., Jarvinen, P., and Isola, J. Amplification of her-2/neu and topoisomerase IIalpha in primary and metastatic breast cancer. Cancer Res., 61: 5345-5348, 2001.

21. Pollack, J.R., Perou, C.M., Alizadeh, A.A., Eisen, M.B., Pergamenschikov, A., Williams, C.F., Jeffrey, S.S., Botstein, D., and Brown, P.O. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat. Genet., 23: 41-46, 1999.

22. Tirkkonen, M., Tanner, M., Karhu, R., Kallioniemi, A., Isola, J., and Kallioniemi, O.P. Molecular cytogenetics of primary breast cancer by CGH. Genes Chromos. Cancer, 21: 177-184, 1998.

23. Jarvinen, T.A., Tanner, M., Barlund, M., Borg, A., and Isola, J. Characterization of topoisomerase IIα gene amplification and deletion in breast cancer. Genes, Chrom. and Cancer, 26: 142-150, 1999.

24. Berger, J.M., Gamblin, S.J., Harrison, S.C., and Wang, J.C. Structure and mechanism of DNA topoisomerase II. Nature, 379: 225-232, 1996.

25. Burden, D.A., and Osheroff, N. Mechanism of action of eukaryotic topoisomerase II and drugs targeted to the enzyme. Biochim. Biophys. Acta., 1400: 139-154, 1998.

26. Fortune, J.M., and Osheroff, N. Topoisomerase II as a target for anticancer drugs: when enzymes stop being nice. Prog. Nucl. Acid Res. Mol. Biol., 64: 221-253, 2000.

27. Holden, J.A. DNA topoisomerase as anticancer drug targets: from the laboratory to the clinic. Curr. Med. Chem. – Anti-Cancer Agents, 1: 1-25, 2001.

28. Withoff, S., Keith, W.N., Knol, A.J., Coutts, J.C., Hoare, S.F., Mulder, N.H., de Vries, E.G. Selection of a subpopulation with fewer DNA topoisomerase II alpha gene copies in a doxorubicin-resistant cell line panel. Br. J. Cancer, 74: 502-507, 1996.

29. Asano, T., An, T., Mayes, J., Zwelling, L.A., Kleinerman, E.S. Transfection of human topoisomerase IIα into etoposide-resistant cells: transient increase in sensitivity followed by down-regulation of the endogenous gene. Biochem. J., 319: 307-313, 1996.

30. Zhou, Z., Zwelling, L.A., Kawakami, Y., An, T., Kobayashi, K., Herzog, C., Kleinerman, E.S. Adenovirus-mediated human topoisomerase IIα gene transfer increases the sensitivity of etoposide-resistant human breast cancer cells. Cancer Res., 59: 4618-4624, 1999.

31. Woessner, R.D., Mattern, M.R., Mirabelli, C.K., Johnson, R.K., and Drake, F.H. Proliferation and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. Cell Growth Differ., 2: 209-214, 1991.

32. Lynch, B.J., Guinee, D.G., and Holden, J.A. Human DNA topoisomerase II-alpha: a new marker of cell proliferation in invasive breast cancer. Hum. Path., 28: 1180-1188, 1997.

33. Jarvinen, T.A., Holli, K., Kuukasjarvi, T., Isola, J.J. Predictive value of topoisomerase II alpha and other prognostic factors for epirubicin chemotherapy in advanced breast cancer. Br. J. Cancer, 77: 2267-2273, 1998.

34. Lehmann, U., Glockner, S., Kleeberger, W., Feist, H., von Wasielewski, R., and Kreipe, H. Detection of gene amplification in archival-cancer specimens by laser-assisted microdissection and quantitative real-time polymerase chain reaction. Am. J. Path., 156: 1855-1864, 2000.

35. Holden, J.A., Perkins, S.L., Snow, G.W., and Kjeldsberg, C.R. Immunohistochemical staining for DNA topoisomerase II in non-Hodgkin's lymphomas. Am. J. Clin. Path., 104: 54-59, 1995.

36. Simmone, N.L., Bonner, R.F., Gillespie, J.W., Emmert-Buck, M.R., and Liotta, L.A. Laser-capture microdissection: opening the microscopic frontier to molecular analysis. Trends in Genet., 14: 272-276, 1998.

37. Wittwer, C.T., Ririe, K.M., Andrew, R.V., David, D.A., Gundry, R.A., and Balis, U.J. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques, 22: 176-181, 1997.

38. Bernard, P.S., Pritham, G.H., and Wittwer, C.T. Color multiplexing hybridization probes using the apolipoprotein E locus as a model system for genotyping. Anal. Biochem., 273: 221-228, 1999.

39. Rasmussen, R. Rapid Cycle Real-Time PCR Methods and Applications. *In*: S. Meuer, C. Wittwer, and K. Nakagawara (eds.), Quantification on the LightCycler, pp. 21-34. Germany: Springer-Verlag, 2001.

40. Glantz, S.A. Primer of biostatistics. *In*: M. Wonsiewicz and P. McCurdy (eds.), Experiments When Outcomes are Measured on a Nominal Scale: McNemar's Test, pp. 314-317. USA: McGraw-Hill, 1997.

41. The International Human Genome Mapping Consortium. A physical map of the human genome. Nature, 409: 934-941, 2001.

42. Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslen, L.A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S.X., Lonning, P.E., Borresen-Dale, A-L., Brown, P.O., and Botstein, D. Molecular portraits of human breast tumours. Nature, 406: 747-751, 2000.

43. Pietras, R.J, Arboleda, J., Reese, D.M., Wongvipat, N., Pegram, M.D., Ramos, L., Gorman, C.M., Parker, M.G., Sliwkowski, M.X., and Slamon, D.J. HER-2 tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. Oncogene, 10: 2435-2446, 1995.

44. Harris, L.N., Yang, L., Liotcheva, V., Pauli, S., Iglehart, J.D., Colvin, O.M., and Hsieh, T.S. Induction of topoisomerase II activity after erbB2 activation is associated with a differential response to breast cancer chemotherapy. Clin. Cancer Res., 7: 1497-1504, 2001.

45. Prost, S. Mechanisms of resistance to topoisomerase poisons. Gen. Pharmacol., 26: 1673-1684, 1995.

46. Fiddes, R.J., Campbell, D.H., Janes, P.W., Sivertsen, S.P., Sasaki, H., Wallasch, C., and Daly, R.J. Analysis of Grb7 recruitment by heregulin-activated erbB receptors revaesl a novel target selectivity for erbB3. J. Biol. Chem., 273: 7714-7724, 1998.

47. Wang, S., Saboorian, M.H., Frenkel, E., Hynan, L., Gokaslan, S.T., and Ashfaq, R. Laboratory assessment of the status of Her-2/neu protein and oncogene in breast cancer specimens: comparison of immunohistochemistry assay with fluorescence in situ hybridization assays. J. Clin. Pathol., 53: 374-381, 2000.

48. Jacobs, T.W., Gown, A.M., Yaziji, H., Barnes, M.J., and Schnitt, J. Specificity of HercepTest in detrmining HER-2/neu status of breast cancers using the United States Food and Drug Administration-approved scoring system. J. Clin. Oncol., 17: 1983-1987, 1999.

49. Gancberg, D., Lespagnard, L., Rouas, G., Paesmans, M., Piccart, M., Di Leo, A., Nogaret, J.M., Hertens, D., Verhest, A., and Larsimont, D. Sensitvity of HER-2/neu antibodies in archival tissue samples of invasive breast carcinomas. Correlation with oncogene amplification in 160 cases. Am. J. Clin. Pathol., 113: 675-682, 2000.

50. Press, M.F., Hung, G., Godolphin, W., and Slamon, D.J. sensitivity of HER-2/neu antibodies in archival tissue samples: potential source of error in immunohistochemical studies of oncogene expression. Cancer Res., 54: 2771-2777, 1994.

Table 1 *Primer and probe sequences for multiplex PCR*

Target	Primers[a]	Probes[b,c]
c-*erb*B-2	GTTGGCATTCTGCTGGTCGT (F) TCCGTTTCCTGCAGCAGTCT (R)	GGATCCTCATCAAGCGACGG -FAM LCR640 - CAGCAGAAGATCCGGAAGTACACG - P
topo IIα	GCGTGTTGAGCCTGAATGGT (F) TCACGCACATCAAAGTTGGGG (R)	GGTGCTGATAAATGGTGCTGAAGG - FAM LCR705 - AATCGGTACTGGGTGGTCCTGC - P
albumin	TCGCCTGAGCCAGAGATTTC (F) CCTGTCATCAGCACATTCAAGC (R)	GTGACAGATCTTACCAAAGTCCACACG - FAM CY5 - GAATGCTGCCATGGAGATCTGC - P

[a] F denotes forward primer, R denotes reverse primer

[b] P denotes phosphate group

[c] FAM indicates fluorescein dye (5-FAM)

Table 2 *Correlation between proliferation and c-erbB-2 status*

c-*erb*B-2 analysis	score	# of samples	topo IIα IHC index[a]
IHC	Positive		
	3^+	23	41
	2^+	22	27
PCR	Positive	27	**42**
	4-7 copies	12	41
	>7-10 copies	8	41
	>10 copies	7	46
	Negative		
	<4 copies	21	23
FISH	Positive	18	**35**
	4-7 copies	3	36
	>7-10 copies	3	43
	>10 copies	12	33
	Negative		
	<4 copies	18	26

[a]Bold type represents average proliferation for all samples scored amplified for c-*erb*B-2 by PCR and FISH

Table 3 *DNA alterations in c-erbB-2 and topoisomerase IIα as determined by PCR*

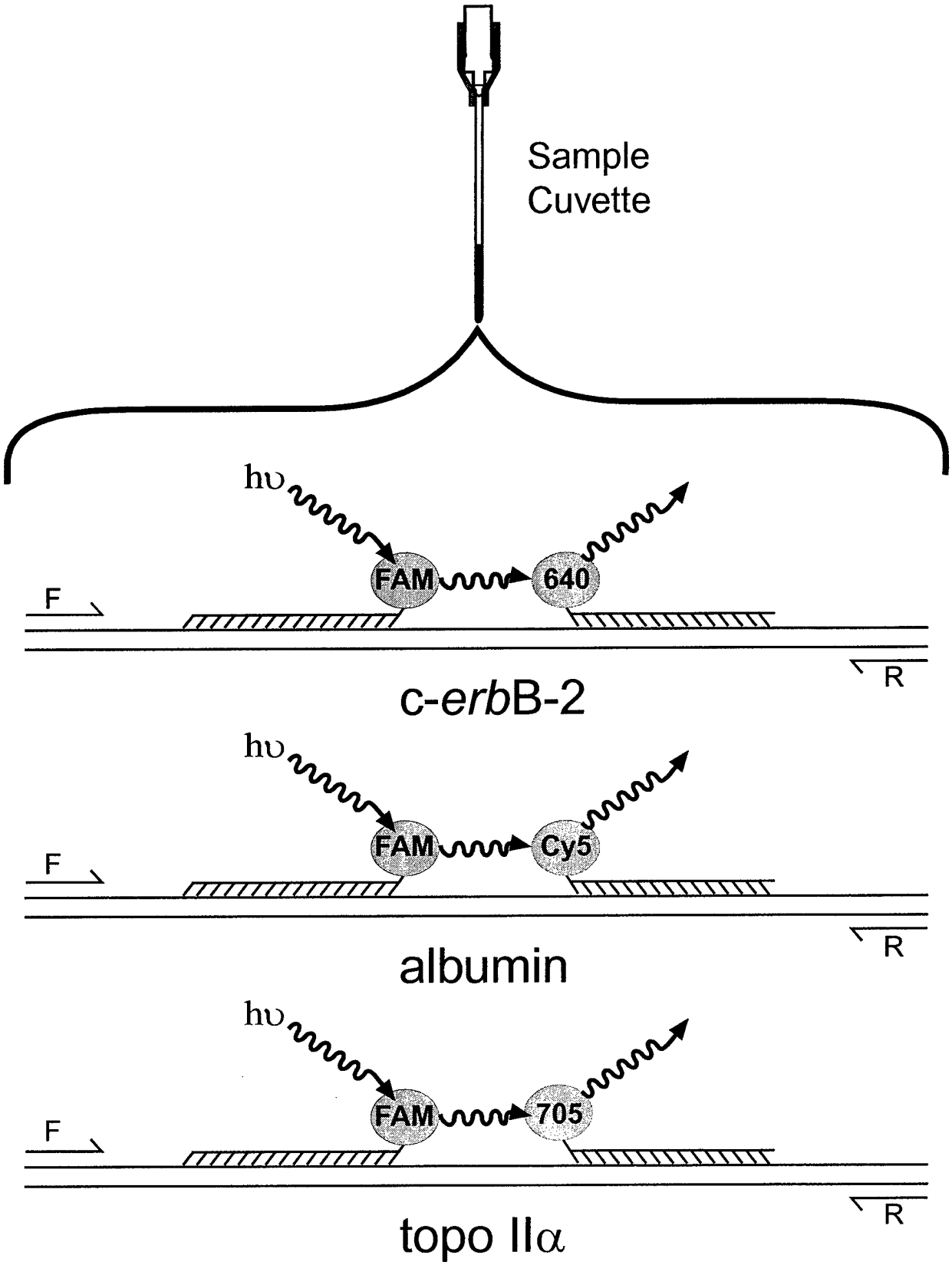
c-*erb*B-2 PCR[a]	topo IIα PCR	
	amplified	deleted
amplified	3/31 (10%)	4/31 (13%)
non-amplified	0/28 (0%)	0/28 (0%)

[a] No tumors were deleted for c-*erb*B-2

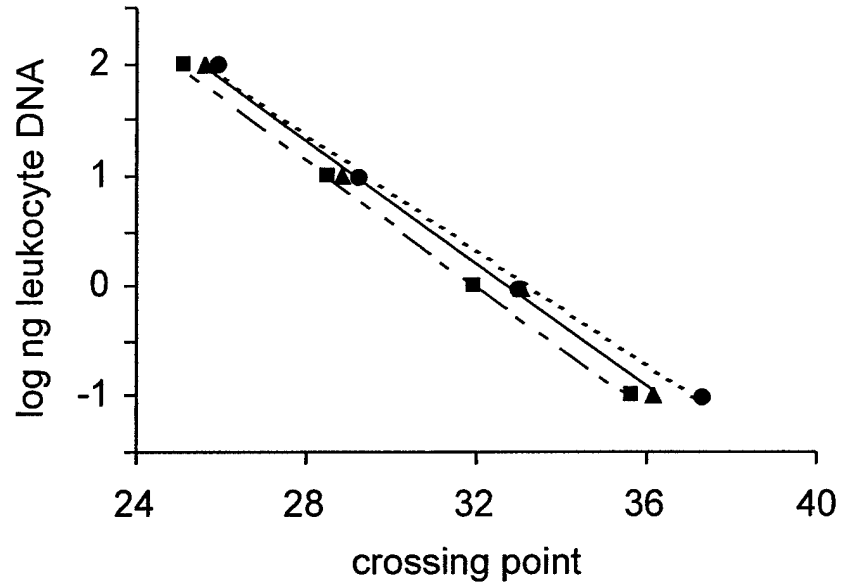
Figure Legends

Fig. 1. A schematic of 3-color multiplexing. Forward (F) and reverse (R) primers simultaneously amplify c-*erb*B-2, albumin and topoisomerase IIα. Once per cycle, during a combined annealing/extension step of PCR, fluorescence is acquired as sequence specific probes hybridize internal to their primer sets. The adjacent hybridization of the fluorescently-labeled probes to their complementary target brings the fluorescein (FAM) donor molecule near (within 1 base) to an acceptor chromophore resulting in fluorescence resonance energy transfer. Each target is monitored in real-time from the distinct emission of its acceptor-labeled probe – LCRed640 (c-*erb*B-2), Cy5 (albumin), and LCRed705 (topoisomerase IIα).

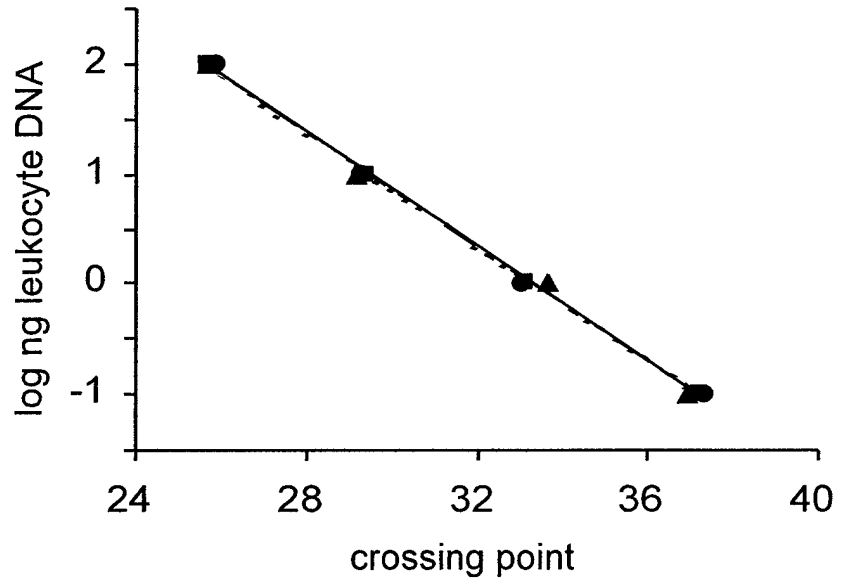
Fig. 2. Raw and corrected standard efficiency curves. Efficiency curves were established within each run using 4 cuvettes with different concentrations of 10-fold serially diluted human leukocyte genomic DNA (100 ng to 0.1 ng). After color compensation of the multiplexed reactions, the amplification curve crossing points (C_ps) for c-*erb*B-2 (●), albumin (■), and topo IIα (▲) were determined. A linear regression line (trend line) through a plot of C_p versus log ng genomic DNA provided an average efficiency for each target in that run (A): c-*erb*B-2 (1.83, -------), albumin (1.93,-----), and topo IIα (1.90, ——). In order to directly compare crossing points between targets, the different efficiencies were then matched for slope and y-intercept (B).


Sample
Cuvette
hυ
FAM
640
F
R
c-erbB-2
hυ
FAM
Cy5
F
R
albumin
hυ
FAM
705
F
R
topo IIα

A


log ng leukocyte DNA
2
1
0
-1
24
28
32
36
40
crossing point

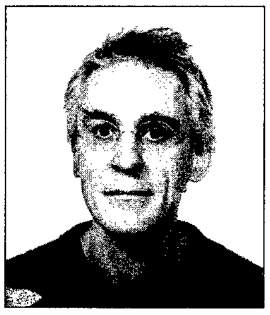
B


log ng leukocyte DNA
2
1
0
-1
24
28
32
36
40
crossing point

DNA Topoisomerases as Anticancer Drug Targets: From the Laboratory to the Clinic

Joseph A. Holden*

Department of Pathology, University of Utah, Salt Lake City, Utah, 84132, USA



Abstract: DNA topoisomerases play important roles in basic cellular biology. Recently they have been identified as the molecular targets of a variety of pharmaceutical agents. Some of the drugs that target the topoisomerases are anticancer drugs. These anticancer drugs work by a novel mechanism of action. They inhibit the topoisomerase molecule from religating DNA strands after cleavage. This leaves a cell with DNA breaks, which if not repaired, become lethal. In other words, these drugs convert the topoisomerase molecule into a DNA damaging agent. This is a stoichiometric relationship. Each anticancer drug molecule has the potential of interacting with one topoisomerase molecule to cause one DNA lesion. The clinical implication of this mechanism of drug action is that sensitivity to topoisomerase targeting drugs should be dependent on high topoisomerase levels. This is clearly true in laboratory systems. With new developments in *in situ* immunohistochemistry, topoisomerase expression can now be easily estimated in human cancers. From this information, it may be possible to predict the sensitivity or resistance of human cancers to topoisomerase targeting anticancer drugs.

A. INTRODUCTION

DNA topoisomerases have fascinated and interested both basic and clinical scientists for several decades. First discovered in 1971, as an enzymatic activity in *E. coli*, which could convert highly negatively supercoiled DNA to its relaxed form [1], enzymes of this sort have been described from organisms on the evolutionary scale ranging from bacteria to humans. These enzymatic activities are essential to resolve topological problems that are inherent in processes involving nucleic acids. Aside from their role in basic cellular reactions, this group of enzymes has now been established as the molecular targets for a variety of antibiotics and anticancer drugs. Over the last few years, there have been many outstanding reviews published on the topoisomerases and the drugs, which target them [2-6]. This review will discuss some newer developments in the field as well as examine the expression of these enzymes in human malignancies and the role that these enzymes may play anticancer drug therapy.

The enzymes are referred to as topoisomerases because they are able to change the topology, or three-dimensional geometry, of DNA molecules without changing the underlying chemical structure of the DNA. They catalyze isomerization reactions between different topological DNA forms. As indicated in (Fig. **1**), these topoisomerization reactions can be studied in the laboratory with the use of circular plasmid DNA. Topoisomerization activities can be observed in extracts of many cell types. Enzymatic activities have been observed which are able to relax supercoiled DNA, catenate and decatenate circular DNA molecules, tie DNA in knots or deknot DNA, and at least in bacteria, activities are present which are able to supercoil relaxed plasmid molecules. It becomes obvious when looking at these reactions, that to carry them out requires cutting and then rejoining DNA strands. This is the basic reaction catalyzed by the topoisomerases. They make a transient DNA break during which the topoisomerase enzyme is covalently attached to the DNA via an active site

*Address correspondence to this author at the Department of Pathology, University of Utah, Salt Lake City, Utah, 84132, USA; Tel: 801-581-2507; Fax: 801-585-3831; E-mail: joe.holden@path.med.utah.edu

1568-0118/01 $20.00+.00

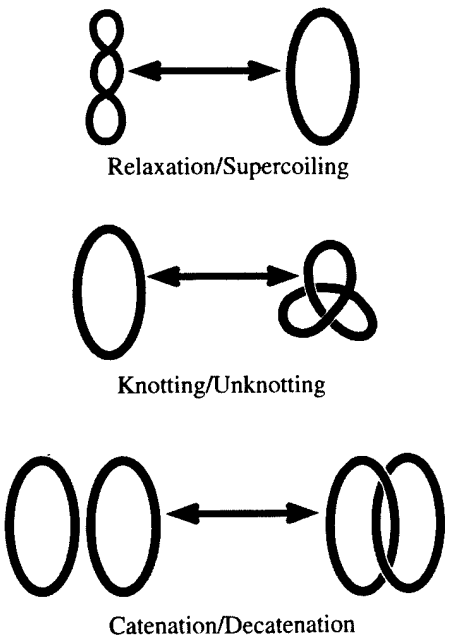


Fig. (1). Topoisomerase Catalyzed Reactions. Circular plasmid DNA molecules, that differ only in their topology, or three-dimensional structure in space, can be interconverted by the topoisomerases. To catalyze these interconversions, the topoisomerases must cut and then rejoin DNA strands.

tyrosine residue, they pass another DNA strand through the transient break in the DNA, and then they reseal (religate) the DNA break. This enzymatic reaction is a concerted cleaving and religating reaction. The transient DNA break formed during the topoisomerization reaction is short lived under normal circumstances. The end result of the reaction is a DNA molecule which is chemically unchanged and covalently closed, but in a different topology.

Some anti-topoisomerase drugs have as their primary mode of action, inhibition of enzymatic activity. Other drugs targeting the topoisomerases interfere with the enzyme's cleavage and rejoining activities and by doing so increase the half-life of the transient topoisomerase catalyzed DNA break. Some of the most clinically useful anticancer drugs are of the latter type and have been referred to as topoisomerase poisons.

B. TYPES OF TOPOISOMERASES

Initially, topoisomerases were simply classified as either a type I or type II enzyme depending on whether they catalyzed their reactions by making transient single strand DNA breaks (the type I enzyme) or transient double strand DNA breaks (the type II enzyme) as shown in (Fig. **2**). This mechanistic description is still correct but has become slightly more complicated with the recent discovery of new members of the topoisomerase family. A topoisomerase III has been described which makes transient single strand DNA breaks and is therefore a type I enzyme, and a topoisomerase IV has been described which makes transient double strand DNA breaks and is therefore a member of the type II enzyme family. The type I enzymes have been further subdivided into type IA and type IB subfamilies based on their reaction mechanism. Type I topoisomerases which form covalent linkages to the 5' end of the DNA break are members of the type IA subfamily and type I enzymes which form covalent linkages to the 3' end of DNA break are members of the type IB subfamily (Fig. **2**). All type II topoisomerases require ATP. Type I topoisomerases do not.

The DNA is covalently linked to the enzyme only during a short period of time during the catalytic cycle. In the cell, this is a transient linkage and readily reversible. The complex of DNA and a covalently bound topoisomerase molecule has been referred to as a "cleavable complex" because

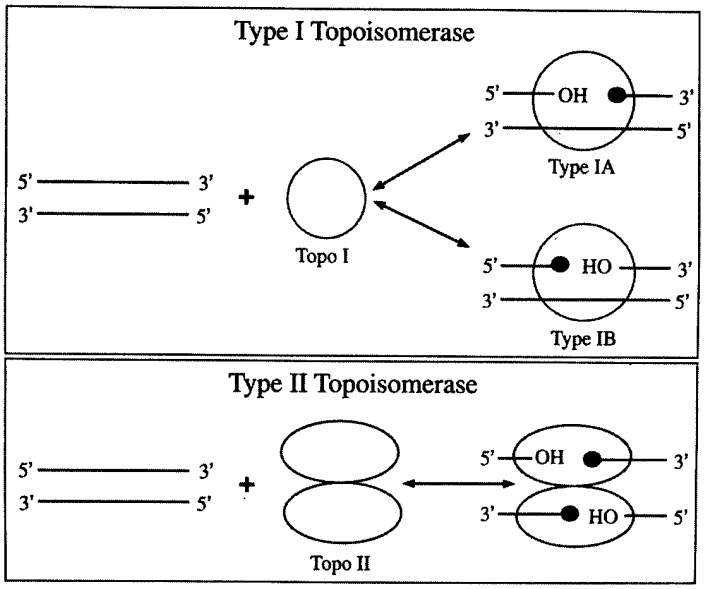


Fig. (2). Type I topoisomerases make single strand DNA cuts and type II topoisomerases make double strand DNA cuts. The topoisomerization reactions carried out by either the type I or type II enzyme involve a transient DNA break. During this reaction, the enzyme becomes covalently linked to the break site via a phosphotyrosine bond. The type I topoisomerases are monomers and are classified as members of the type IA subfamily if the enzyme becomes covalently linked to the 5' end of the DNA break. Type I enzymes that become covalently linked to the 3' end of the break site are classified as members of the type IB subfamily. Type II topoisomerases make double strand DNA breaks with a four base pair overhang. The enzyme becomes covalently linked to the 5' ends of the break site. The diagram is depicting eukaryotic topo II which is a homodimer. The bacterial type II enzymes function in a similar fashion but the enzymes are tetramers.

in the laboratory, if it is treated with denaturants and proteinase digestion, the topoisomerase molecule becomes permanently linked to the DNA molecule. The added proteinase then digests the topoisomerase molecule away from the DNA. The topoisomerase induced DNA break is never religated under these laboratory conditions and therefore the DNA is left with strand breaks, which are detected by gel electrophoresis [7].

C. PROKARYOTIC TOPOISOMERASES

1. *E. coli* DNA Topoisomerase I

In order to understand the relationship among eukaryotic topoisomerases and to gain insight into how different classes of drugs may target these enzymes, it is useful to have an understanding of the types of enzymes that exist in prokaryotes and the drugs that target them. The first description of a topoisomerase was published in 1971 [1]. The protein discovered in *E. coli*, originally called the omega protein, was able to relax negatively supercoiled plasmid DNA. The enzyme became known as *E. coli* topoisomerase I (topo I). The enzyme is a monomer of 97 kDa (kilodaltons) [8] and is coded for by the *topA* gene [9]. It has a preference for binding at single strand regions of DNA [10]. This explains why the enzyme readily relaxes negatively, but not positively supercoiled DNA, as single stranded areas tend to be present in negatively supercoiled (underwound) DNA but not in positively supercoiled DNA. Interestingly, if a single stranded region is incorporated into a positively supercoiled circular plasmid, *E. coli* topo I is now readily able to relax the DNA [11].

The crystallographic structure of a 67 kDa fragment of *E. coli* topo I provides insight into the enzyme's mechanism of action and substrate preference. The enzyme is composed of four molecular domains and a large 27.5 Å hole [12]. The diameter of the hole and the charge distribution of the amino acids residues composing it are appropriate for the accommodation of either a single or double strand DNA molecule. The active site tyrosine at position 319 is located in domain III. After binding to a single stranded DNA region, a

nucleophilic attack by the hydroxyl group of tyrosine 319 on the DNA phosphodiester linkage, covalently binds the enzyme to the 5' phosphoryl end of the DNA break. The covalent linkage of the enzyme to the 5' phosphoryl end of the DNA defines *E. coli* topo I as a member of the type IA subfamily. The 3' hydroxyl end of the broken DNA becomes non-covalently bound to the enzyme in domain III and/or IV. Both ends of the broken DNA strand are associated with the enzyme. Thus the enzyme does not act like a "swivel" allowing for free rotation about the DNA axis as its eukaryotic counterpart may, but rather "bridges" the DNA break. After a conformational change in which domain II and III move away from domain I, the large 27.5 Å hole is now available to accommodate a second DNA molecule which passes through the enzyme bridged break and enters the large 27.5 Å cavity. The reaction is completed when the initial DNA break is resealed and both DNA strands exit the molecule [13,14].

Previous work suggested that the major function of *E. coli* topo I was to help regulate the overall steady state of superhelical density of DNA in the cell [15,16]. Although this may still be an important function of this protein, in view of recent data [17], it might also be appropriate to view *E. coli* topo I as a transcription factor. During transcription, positive supercoils accumulate in front of the transcription machinery, which can be removed by gyrase, and negative supercoils accumulate behind. It may be that a major function of *E. coli* topo I is to relax underwound DNA which accumulates behind an active RNA polymerase. Interestingly, the C-terminus of *E. coli* topo I contains regions that show sequence homology to the zinc binding domains of several types of transcription factors [18].

2. *E. coli* DNA Topoisomerase III

The identification of a second type I enzyme in E. coli was made in *topA* deletion strains. It was observed that such deletion strains still contained an activity capable of relaxing negatively supercoiled DNA. The activity was purified and referred to as *E. coli* topoisomerase III (topo III) [19]. Subsequent studies indentified this protein as a factor necessary for the separation of replicating plasmids [20]. The gene coding for topo III is referred to as *topB*. It is capable of coding for a 74 kDa protein [21]. Topo III is a member of the type IA subfamily and as expected, both topo I and topo III share extensive sequence homology. The homology is most noted in the center of the molecule but becomes divergent in the amino and carboxyl terminal ends [19]. However, in spite of their homology, their *in vivo* functions may be quite different. Topo III is more efficient at decatenating DNA molecules than relaxing them while the efficiency of topo I in these reactions is the reverse. These properties suggest that the major role of topo III may be to decatenate daughter chromosomes at the end of a round of DNA replication. Consistent with this interpretation are data which show that topo III is able to support DNA chain elongation but topo I is not [22]. It is important to note that because topo III is a type I enzyme and is therefore only capable of making transient DNA single strand breaks during catalysis, decatenation of double strand DNA molecules by topo III requires a small nick or gap in one of the DNA strands.

Another interesting difference between topo I and topo III is that topo III has been shown to catalyze RNA strand passage, unlike topo I [23]. Whether this property of topo III is physiologically significant is not yet clear but suggests that perhaps topo III is involved in topological interconversions of RNA as well as DNA.

The crystallographic structure of topo III indicates that the sequence homology with topo I is reflected with significant structural homology as evident from the three dimensional structure of the protein. Like topo I, topo III is found to have 4 domains and a large, approximately 25 Å hole which can accommodate a DNA molecule. Similar to topo I, the active site tyrosine at residue 328 is located in domain III. The structure suggests that topo III is mechanistically quite similar to topo I [24]. Interestingly, topo III has a large insertion loop in domain IV that is not present in topo I. The insertion loop has been referred to as the decatenation loop because if it is absent, the enzyme will no longer carry out catenation or decatenation reactions. The exact mechanism in which the

decatenation loop facilitates such a reaction is not yet clear.

As will become apparent during this review, the topoisomerases are targets of a wide number of antibiotics and anticancer drugs. However, no drug has yet been described which targets the topoisomerases of the Type IA subfamily.

3. *E. coli* DNA Gyrase and DNA Topoisomerase IV

The type II topoisomerases are represented in *E. coli* by DNA gyrase and topoisomerase IV (topo IV). Unlike the type I topoisomerases, the type II enzymes all require ATP for catalysis. As type II enzymes, these molecules carry out their topoisomerization reactions by making transient double strand DNA breaks to allow for DNA strand passage (Fig. **2**). The transient double strand break occurring during the reaction is a staggered break with a 4 base pair overhang. The enzyme becomes covalently linked to the DNA backbone by a phosphotyrosine bond. Since each strand of the cleaved DNA is covalently bound to the enzyme, free rotation about the helical axis does not occur, and like topo I, the enzyme "bridges" the transient break. Interestingly, some structural similarities between these otherwise unrelated type IA and type II topoisomerases have recently been identified [25].

DNA gyrase was discovered prior to topo IV [26]. As a type II enzyme, it requires ATP. DNA gyrase is a heterotetramer composed of two A and two B subunits to yield an A_2B_2 holoenzyme. The A subunit is the product of the *gyrA* gene and has a molecular weight of 97 kDa. The B subunit is the product of the *gyrB* gene and has a molecular weight of 90 kDa [27].

Gyrase is unique among topoisomerases because it is able to supercoil DNA. Incubation of relaxed circular DNA with gyrase and ATP leads to the production of negatively supercoiled DNA. The formation of supercoiled DNA from relaxed DNA suggests that strand passage by gyrase must have direction, otherwise strand passages yielding slightly supercoiled substrates would eventually be cancelled out by strand passages in the opposite direction leading to relaxation. The enzyme is able to accomplish a direction to strand passage by wrapping the DNA around the enzyme in a right-handed supercoil of 135 base pairs, something that the eukaryotic enzyme can not do [28,29]. When catalyzing strand passage in this matter, direction is accomplished and supercoiling proceeds. This supercoiling reaction is unique to prokaryotes. No active supercoiling activity has yet been described in higher organisms.

Topoisomerase IV was discovered in partition mutants of *E. coli*. These are mutants that are unable to segregate daughter chromosomes into daughter cells [30]. This results from a failure to decatenate DNA after replication. Two genes are responsible for the phenotype and are referred to as *par E* and *par C*. The protein coded for by the *par C* gene is homologous to the gyr A subunit and the protein coded for by the *par E* gene is homologous to the gyr B subunit. Like gyrase, topoisomerase IV is a heterodimer and consists of two *par C* encoded subunits and two *par E* encoded subunits giving a molecular structure for the enzyme of C_2E_2. Unlike gyrase, the enzyme does not wrap DNA around the enzyme molecule in a right handed coil and therefore is unable to supercoil DNA [31]. The enzyme is a potent decatenase and plays a critical role in decatenating DNA after replication to allow proper partitioning of daughter chromosomes.

The division of labor between gyrase and topo IV has been subject to debate. Early studies suggested that the partitioning of DNA between daughter cells was taken care of by gyrase [32], but this was before topo IV was known to exist. With the discovery of topo IV, and that it was an active decatenase and was unable to supercoil DNA, it was believed that topo IV was the primary decatenase while gyrase functioned to remove supercoils which accumulate during the elongation phase of DNA replication [27]. It now appears that topo IV can also function to remove supercoils that occur in front of the replication fork [33] so these two type II topoisomerases may share in the work of DNA chain elongation. In addition, the combina-tions of the supercoiling activity of gyrase and the relaxing activities of topo I and

topo IV probably act in concert to maintain an overall negative superhelical density in the bacterial cell [34].

Recent crystallographic structures of gyrase fragments have provided an insight into the enzyme's mechanism of action [35]. The ATPase activity is located in the gyrB subunit. Each gyrB subunit is a crescent shaped protomer and the subunits dimerize upon ATP binding. The gyrB protomers outline a central hole of about 20 Å. The hole is able to accommodate a DNA double helix due to its positive charge and size. Likewise, a crystallographic structure of the N-terminal fragments of the gyrA dimer shows that both gyrA protomers also outline a central hole. The active site tyrosines are located in the gyrA dimer. Putting these structures together suggests that the enzyme works like a molecular clamp as shown in (Fig. **3**). The DNA segment to be cleaved (the G or gate segment) is bound by the gyrA dimer. The arms of the clamp are exemplified by the gyrB protomers. They are open and ready to capture the DNA segment (the T segment) that will be transported thought the gate. Once the T segment is captured, the clamp closes around the T segment after ATP hydrolysis, the G segment is cleaved, and the T segment is transported through the break. The G segment is then religated and both DNA segments exit the topoisomerase.

It might be anticipated that topo IV would have a similar mechanism of action and molecular structure however crystallographic data for topoisomerase IV have not yet been published.

D. DRUGS TARGETING THE PROKARYOTIC TYPE II TOPOISOMERASES

DNA gyrase and topoisomerase IV are the molecular targets of two distinct groups of antibiotics; the coumarins (novobiocin) and the quinolones (ciprofloxacin). These two classes of drugs work by different mechanisms and are analogous to the mechanism of action of the two broad classes of anticancer drugs that target the eukaryotic enzymes. In general, topoisomerase targeting drugs as antibiotics or anticancer drugs either, inhibit the catalytic activity of the enzyme, or impair the ability of the enzyme to religate DNA after cleavage. Although drugs of the latter type necessarily inhibit catalysis as well, it is thought that the inhibition of DNA religation after cleavage leaves a cell with drug stabilized DNA breaks which are eventually converted into chromosomal breaks which result in cell death.

An example of the first type of antibiotic is represented by the coumarins. These drugs inhibit the ATPase activity of gyrase. Because the coumarin antibiotics bind to a site that overlaps that of ATP on the B subunit, they are competitive inhibitors [36].

Representative of the second class of topoisomerase targeting drugs are the quinolones. Theses drugs interact with gyrase or topoisomerase IV and their DNA substrates and prevent the topoisomerase from religating DNA after cleavage [37, 38]. This leaves the cell with double strand DNA breaks. If these breaks are not repaired, they interact with other intracellular proteins and become converted into irreversible double strand breaks that lead to cell death.

Double strand DNA breaks are a normal intermediate in the topo II reaction mechanism. However, they are short-lived. The overall effect of the quinolones, by inhibiting DNA religation after topoisomerase cleavage, is to increase the half life of this normal reaction intermediate. This increases the likelihood of encounters with other cellular proteins that may result in irreversible DNA strand breakage. This mechanism of antibacterial drug action against prokaryotic type II topoisomerases is reminiscent of the mechanism of action of several anticancer drugs that are targeted against human topoisomerase II. In both instances, the drug's primary mechanism of action is not simply to inhibit topoisomerase enzymatic activity, but rather to convert the enzyme to a DNA damaging agent.

E. EUKARYOTIC TOPOISOMERASES

1. Eukaryotic DNA Topoisomerase I

The topoisomerase group of enzymes is also well represented in eukaryotes and these proteins

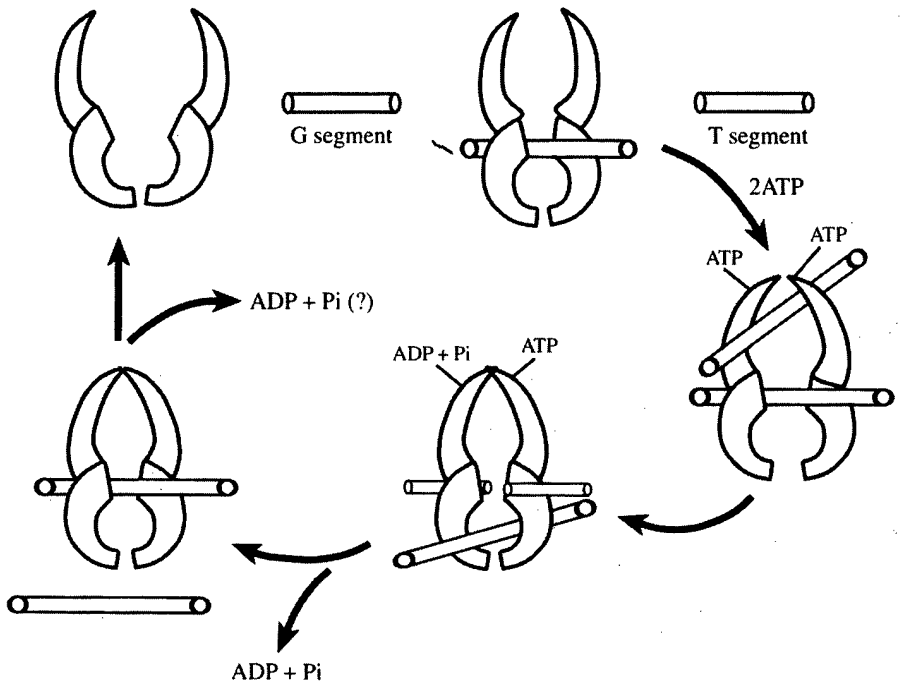


Fig. (3). Topoisomerase II functions as a molecular clamp. The model indicated is based on the crystallographic structures of bacterial gyrase and yeast topo II as well as on the kinetic analysis of yeast topo II. Both the prokaryotic and eukaryotic type II topoisomerases show sequence homology. The bacterial topo II is a heterotetramer. For bacterial gyrase, the arms of the clamp represent the gyr B subunits and they are open and ready to capture the DNA segment (T segment) that will be transported through the enzyme catalyzed DNA break. The DNA segment which will be cleaved by the enzyme is referred to as the gate segment (G segment) and it is bound by the gyr A subunits which contain the active site tyrosine. After binding ATP, the gyr B arms clamp around the T segment. The gyr B subunit contains the ATPase activity. The first ATP is hydrolyzed as the G segment is broken and the T segment is passed through the break. The first ADP and Pi are then released. At some later point in the reaction, the DNA strands are released from the enzyme and the second ATP is hydrolyzed. The precise point in the reaction where the second ADP is released is not entirely clear and this fact is indicated by the question mark. The enzyme is then ready to begin another round of catalysis. The eukaryotic enzyme apparently arose from gene fusion of an earlier ancestor so that the ATPase activity and the active site tyrosines are not on separate polypeptides as in the bacterial enzyme, but reside on the same subunit. The mechanism of catalysis is probably quite similar.

have been extensively studied from yeast, drosophila, and mammalian cells.

Eukaryotic DNA topoisomerase I is a member of the type 1B family because when it cleaves DNA it becomes covalently attached to the 3' DNA end of the break site, unlike bacterial topo I, which becomes attached to the 5' DNA terminus. The gene for the human enzyme is located on chromosome 20 [39]. It is a large gene of about 85 Kb (kilobases) and contains 21 exons [40]. The gene codes for a protein of about 100 kDa. The enzyme exists as a monomer. Two topo I pseudogenes have also been described, one on chromosome 1 and the other on chromosome 22 [41]. Eukaryotic topo I does not share homology with *E. coli* topo I and carries out its topoisomerization reactions in a different manner. Unlike the bacterial enzyme, eukaryotic topo I probably does not "bridge" the enzyme induced single strand DNA break but may allow free, or partially free, rotation about the DNA axis. It acts more like a swivel. It prefers to bind double stranded DNA rather than single stranded DNA [10,42]. These biochemical properties help explain why eukaryotic topo I readily relaxes both positive and negative supercoiled plasmids.

Eukaryotic topo I is located in areas of active RNA transcription and may function ahead of the transcription apparatus to relieve superhelical stress generated during mRNA synthesis [43-45]. A role for the enzyme in the synthesis of rRNA has also been suggested as topo I has been shown

to interact with RNA polymerase I [46] and has recently been shown to be present during ribosomal gene activation occurring during embryogenesis [47]. In addition, the enzyme has been observed to associate with mitotic chromosomes [48] and may play a structural role in chromosome organi-zation [49]. The enzyme is essential for proper embryonic development in drosophila [50] and in mice [51]. Surprisingly, in spite of these many important functions, in yeast, topo I is not necessary for survival which suggests that other cellular proteins may have overlapping roles and probably are able to substitute in topo I's absence [52].

2. Eukaryotic DNA Topoisomerase III

For many years it was thought that eukaryotic topo I was the only type I topoisomerase in higher organisms. This is incorrect as a new type I enzyme, more similar to the *E. coli* type I topoisomerases than to eukaryotic topo I, has been readily documented to exist in a number of higher organisms. This topoisomerase was first described in yeast and identified as the product of a gene whose function was necessary for proper recombination frequency [53]. Mutations in this gene result in hyper-recombination events. Because of this, the gene was first referred to as *edr1-1* for enhanced delta recombination. However, sequencing of the gene revealed that it coded for a 74 kDa protein which had 21.5% amino acid homology with *E. coli* topo I and 44% amino acid homology with *E. coli* topo III. It was therefore referred to as the *TOP3* gene in *Saccharomyces cerevisiae*. Clearly, a type IA enzyme was now documented to exist organisms other than bacteria.

As predicted, biochemical studies of yeast topo III revealed that the enzyme has more in common with the bacterial type I topoisomerases than with eukaryotic ones. Although the enzyme has only a weak relaxing activity, it prefers single strand regions. Like its *E. coli* counterparts, yeast topo III will not effectively relax positively supercoiled substrates. The relative weak relaxation activity of topo III suggests that its primary function may not be in regulating the superhelical density in the cell [54].

It is now clear that type IA topoisomerases do not exist solely in bacteria and yeast but are more universal than initially thought. Although it took seven years after the description of yeast topo III, a similar enzyme was eventually found to exist in drosophila [55], mice [56,57], and human cells [58,59]. In higher organisms topo III exists as alpha and beta isoforms. The human topo III-alpha isoform maps on chromosome 17p11.2-12 and the human topo III-beta isoform is located within the immunoglobulin lambda gene [60]. The function of topo III in higher organisms may be important for normal embryological development [61] and during meiosis [56,62].

The alpha and beta isoforms of topo III, being members of the type IA family, require single stranded DNA regions for optimal activity [55, 63, 64, 65]. Such single stranded regions may form during the interaction of helicases with DNA and most interestingly, it appears that topo III interacts specially with helicases of the RecQ family [66, 67, 68]. For example, Bloom's syndrome is a genetic disorder in which affected individuals show skin rashes and an increased frequency of leukemias. Cells from these patients show hyper-recombination events and contain mutations in the *BLM* gene. The product of the *BLM* gene is now known to be a member of the RecQ family of helicases and has recently been shown to interact with topo III-alpha [69, 70]. These intriguing findings suggest that the topo III enzymes may function in association with helicases in recombination or decatenation events. As already mentioned, the analogous enzyme in *E. coli*, (*E coli* topo III), functions as a decatenase and the yeast counterpart (yeast topo III) was discovered because its absence led to an increase in recombination between repeated sequences. Because the lack of topo III leads to genetic instability that might increase the incidence of malignant transformation, the interesting possibility that the topo III gene may function as a tumor suppressor gene has recently been suggested [69].

Drugs targeting the topoisomerases of the Type IA family have not been described. Therefore, whether these new topoisomerases in human cells may serve as drug targets for antitumor agents

remains to be determined. Topo III has been observed to be expressed in a wide range of normal and neoplastic tissues [71] so even if drugs were available to target the enzyme, they may not show any therapeutic selectivity against malignant cells.

3. Eukaryotic DNA Topoisomerase II

Eukaryotic topoisomerase II (topo II) is a homodimer of identical subunits. In yeast and drosophila only one form of the enzyme exists while in human cells, both an alpha and beta isoform are present [72]. Because invertebrates apparently have only one form of topo II, the separate functions of human alpha and beta isoforms are presumably carried out by a single enzyme in lower organisms.

The human alpha isoform is homodimer with a subunit molecular weight of 170 kDa and the human beta isoform is a homodimer with a subunit molecular weight of 180. The gene for human topo II-alpha is located on chromosome 17 [73] and the gene for human topo II-beta is located on chromosome 3 [74]. Both isoforms share extensive sequence and amino acid homology, which suggests they may have arisen from a duplication of an ancestral gene [75].

Although eukaryotic topo II has an ATP requirement, the enzyme has never been shown to supercoil DNA. The activity of the alpha isoform is necessary to untangle intertwined chromosomes after DNA synthesis to allow for proper chromosomal segregation into daughter cells. The ATP requirement probably provides the energy necessary for the enzyme to control the topology of DNA below that which would be expected at thermodynamic equilibrium [76]. Knotted and catenated DNA structures, which would normally be present at equilibrium, could provide a hindrance to chromosomal segregation. It appears that topo II may use the energy of ATP to unknot and decatenate DNA to an untangled state below that which would expected to form at equilibrium. The untangled DNA would then allow for efficient separation of chromosomes [77]. The enzyme may carry out this role as a component of the nuclear matrix. Topo II is one of the major proteins isolated from the nuclear scaffold where it may also help to anchor chromosomal loops [78]. Thus the enzyme may play a structural as well as a catalytic role in the cell. The precise biological role of topo II-beta is not entirely known but recent data suggests an important role for this enzyme in normal neuromuscular development [79]. Interestingly both human isoforms have been shown to function with RNA containing substrates but whether this *in vitro* reaction has physiological significance remains to be determined [80].

Like the prokaryotic type II enzymes, eukaryotic topo II makes a transient double strand break in order to carry out its topoisomerization reactions. During the formation of the double strand break, the active site tyrosine from each subunit becomes covalently attached to the break site through a 5'phosphotyrosine linkage. The breaks between the strands are not directly opposite each other but rather are separated by a four base pair overhang. Upon hydrolysis of ATP, strand passage occurs. After DNA strand passage, the break is sealed by religation.

Recent kinetic data indicate that the type II topoisomerization reaction catalyzed by the eukaryotic enzyme is a sequential one. Each topo II holoenzyme binds two ATP molecules. One ATP is hydrolyzed rapidly and the DNA segment to be transported (the T segment) is passed through the DNA strand containing the double strand break (the G segment). The second ATP is hydrolyzed only after the first ADP has been released from the enzyme [81].

The ATPase activity and active site tyrosine, which are on separate subunits in the bacterial type II topoisomerase, are on the same subunit in the eukaryotic enzyme. The N-terminal portion of eukaryotic topo II contains the ATPase activity and is homologous with the B subunit of gyrase. The central portion of eukaryotic topo II contains the active site tyrosine and is homologous with the A subunit of gyrase [82]. This suggests that eukaryotic topo II may be the result of gene fusion from an earlier ancestor. The C-terminus of eukaryotic topo II is not homologous with the bacterial enzyme. It contains sequences that probably inter-

act with DNA and localize the protein to the nucleus [83,84]. The alpha and beta human topo II isoforms diverge the most in the C-terminus.

Structural and mechanistic studies suggests that eukaryotic topo II functions like a molecular clamp, similar to bacterial gyrase [35]. As shown in (Fig. **3**), prior to DNA or ATP binding, the enzyme clamp is open. The G segment binds first near the center of the enzyme in the area of the active site tyrosines. The T segment is then captured by the N-terminal arms and in the presence of ATP, the arms dimerize and clamp around the T segment. As the first ATP is hydrolyzed, the G segment is broken by a transesterification reaction with the active site tyrosines, and the T segment is transported through the break in the G segment. The first ADP then is released from the enzyme. At a later stage in the reaction, the G segment is resealed, both DNA strands are released from the enzyme and the second ATP is hydrolyzed. The enzyme clamp reopens to prepare for another round of catalysis.

F. TOPO I TARGETED CHEMOTHERAPY

1. Camptothecin: Mechanism of Action

One of the more exciting developments in clinical oncology has been the identification of the topoisomerases as the molecular targets of a variety of anticancer drugs. This has allowed new insights into the mechanisms of patient response to chemotherapy as well as stimulated a rational search for, and design of, new agents that may find clinical use by targeting these proteins. Presently there have been described a wide variety of drugs which interact with eukaryotic topo I, many of which have anticancer properties. These consist of a variety of natural products and antibiotics. The list of topo I interacting drugs is becoming longer every year and have been the subject of two recent reviews [3,5].

The drugs targeting topo I that are now currently in clinical use are derivatives of camptothecin (Fig. **4**). Camptothecin was isolated in 1966 from the tree *Camptotheca acuminata* that is native to China [85]. Although in early studies, the drug had anticancer properties, clinical trials revealed unacceptable toxicity and further development was stopped. In 1985 it was discovered that topo I was the molecular target of camptothecin [86]. Further studies indicated that topo I is the sole molecular target of the camptothecins. Much of this information has come from studying topoisomerase model systems in yeast. Plasmids containing the human topo I gene under control of a galactose promoter have been developed for laboratory study. With these plasmids, expression of the human enzyme in yeast can be turned on or off simply by the addition of galactose. It has been clearly shown that turning on the expression of the human enzyme in yeast results in sensitivity of the cell to camptothecin and turning off the expression of the human enzyme results in drug resistance [87]. Mutations in topo I confer resistance to camptothecin [88, 89]. Additional studies have indicated that overexpression of the yeast topo I in human cells sensitizes these cells to camptothecin as well and confirms the viability of using yeast as a model system to study topoisomerase anticancer drug interactions [90,91]. Camptothecin also does not intercalate into DNA where it might inhibit a variety of DNA processing enzymes, and the drug also does not interact with topo II. All of these data support the interpretation that topo I is the sole molecular target of the camptothecins and that sensitivity of a cell to these drugs is dependent on elevated levels of topo I.

The recent crystallographic and molecular modeling studies of human topo I provide an insight into the mechanism of camptothecin action and why cellular sensitivity to camptothecin should depend on enzyme levels. Although the sequence specificity of topo I action is fairly non-specific, camptothecin induced topo I dependent DNA cleavages generally show a G residue in the +1 position of the cleaved strand. The crystal structure of topo I in complex with DNA, shows how the camptothecin molecule could insert itself into the active site of the enzyme and displace the 1+ G out of the DNA duplex [92]. By displacing the 1+ G, the ends of the cleaved DNA strand are no longer in alignment and the enzyme is prevented from efficiently religating the DNA after cleavage. An equally plausible mechanism to explain the interaction of camptothecin with DNA

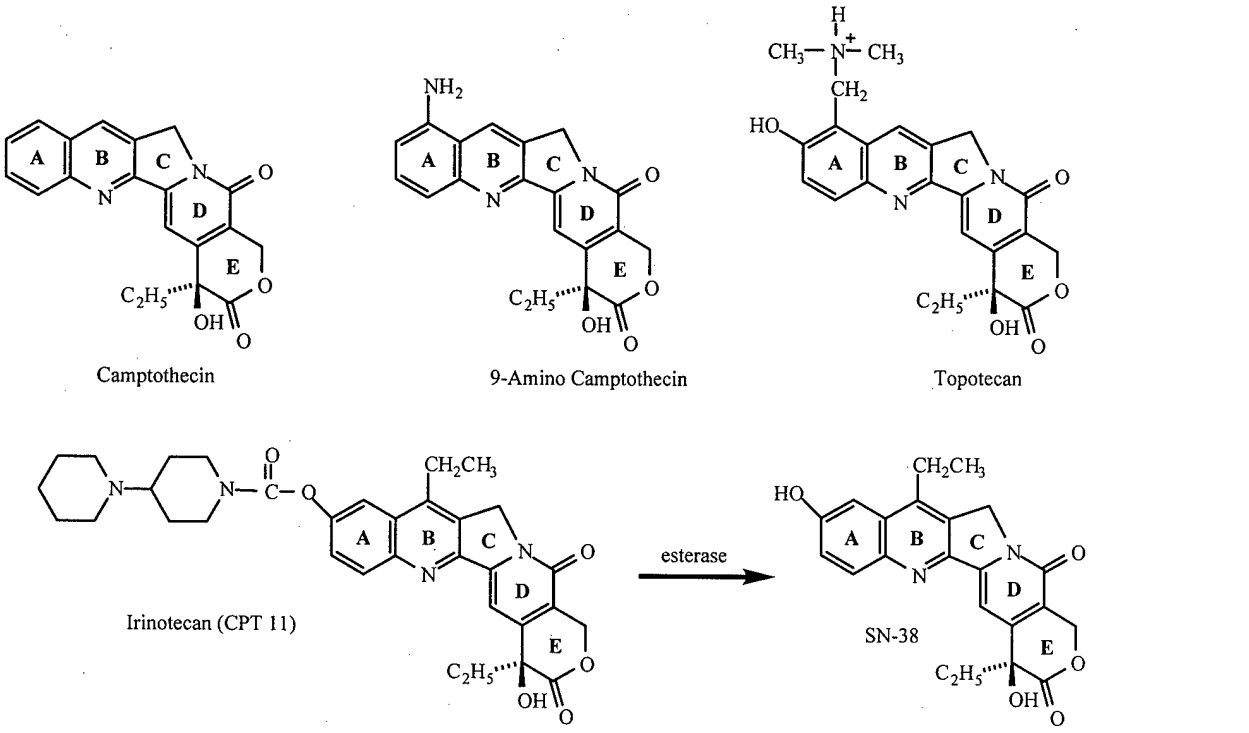


Fig. (4). Molecular structures of camptothecin and derivatives. The activity of camptothecin against topo I requires an intact lactone ring. The carboxylate analog is inactive. Irintotecan is a pro-drug that requires hydrolysis to SN-38 for activity. Irinotecan is active against colorectal cancer and topotecan shows activity against ovarian cancer. Nine-amino camptothecin has not yet been shown to have significant activity against human malignancies.

and topo I has been hypothesized from molecular modeling [93]. This mechanism is a drug "stacking" mechanism in which the camptothecin molecule interacts with topo I and the 1+ purine base but does not require the displacement of the purine or rotation of the camptothecin ethyl group. The importance of the planar structure of the camptothecin, the requirement of an intact lactone E ring, and the requirement that the 20 hydroxyl group be present in the S isomeric configuration are all accommodated by specific interactions with the enzyme in these models. In addition, mutations of topo I, which are known to affect activity and drug response, generally affect amino acid residues that surround the active site. By preventing efficient religation after cleavage, the camptothecin drugs prolong the half life of the normal cleaved DNA intermediate. This act alone probably does not result in cell death, but when the drug stabilized single strand break encounters a replication fork, the single DNA strand break is converted into a double DNA strand break which kills the cell [94]. Recent data suggest that there may be an asymmetry to this process. Camptothecin induced DNA breaks are probably converted into double strand DNA breaks only when they occur on the leading strand during DNA replication [95]. The drugs therefore work by a novel mechanism. They convert the topo I molecule into a DNA damaging agent. This is a stochiometric relationship. The combination of one topo I molecule and one camptothecin molecule results in one DNA strand break. Thus there is the potential for one DNA break for every topo I molecule in the cell. This is why the sensitivity of cells to the camptothecins should be dependent on high levels of topo I. The more topo I, the more DNA damage and the more chance of cell death. This is clearly true in experimental systems. It leads to the interesting hypothesis that the sensitivity of human cancers to topo I targeting anticancer drugs may be dependent on tumor levels of enzyme.

2. Topotecan and Irinotecan

As seen from the molecular structure of the human enzyme, the intact lactone ring of the

camptothecin molecule is necessary for a proper fit into the active site of the enzyme. Unfortunately, the lactone ring is readily hydrolyzed to the carboxylate form at physiological pH. The carboxylate form is inactive. In addition, camptothecin is fairly insoluble. These two problems have been addressed in both topotecan and irinotecan. Topotecan is finding use against ovarian cancer [96]. For irinotecan, recent randomized clinical trials have shown that irinotecan therapy for patients with fluorouracil resistant metastatic colorectal cancer provides for a better quality of life and for prolonged survival when compared to supportive care alone [97,98]. In another recent randomized clinical trial, the combination of irinotecan, fluorouracil and leucovorin was found to be superior to either the combination of fluorouracil and leucovorin or to irinotecan alone, in providing for an increased response rate and increased survival time for patients with metastatic colon cancer [99]. Irinotecan is a pro-drug that needs to be hydrolyzed to its active metabolite, SN-38, as the parent compound is inactive (Fig. **4**).

Topotecan and irinotecan are more water soluble than camptothecin and their lactone forms are more stable in serum than camptothecin. The greater stability of topotecan and irinotecan in serum may rely on the addition of substituents on the 7 and 9 positions. These interfere with binding of their carboxylate forms to human serum albumin [100,101]. Human serum albumin has been show to preferentially bind the carboxylate form of camptothecin thus shifting the lactone-carboxylate equilibrium present in serum, in favor of the inactive carboxylate molecule. The resistance of topotecan and irinotecan in binding to human serum albumin results in a greater amount of the active lactone form in serum. Interestingly, the fact that mouse serum albumin does not bind the carboxylate forms as well as human serum albumin, is perhaps one reason the camptothecins appear much more active against neoplasms in mice [102] than in humans [103].

Additional camptothecin derivatives are currently being synthesized and evaluated. Some of these are able to stabilize the transient topo I catalyzed DNA break to a greater extent then either topotecan or SN-38. Particularly exciting are camptothecin related compounds which contain substituents at the 10,11 and 7 positions such as 10,11-methylenedioxy-20(*S*)-camptothecin and 7 – chloromethyl-10, 11-methylenedioxy-20(*S*)-camptothecin. These compounds are more potent in producing topo I induced strand breakage than camptothecin and this effect may be related to their ability to prolong the half life of the topo I mediated DNA break [104]. Glycinate esters of some of these compounds have recently been synthesized [105]. The glycinate esters have increased water solubilities, are readily hydrolyzed to active drugs at physiologic pH, and show less inhibition of acetylcholinesterase than does camptothecin. Treatment with these compounds could lead more tolerable side effects and to a persistence of the DNA break in a tumor cell which might increase the chance of tumor cell killing. Clinical trials with some of these newer agents will be necessary to determine how effective they are.

3. Tumor Expression of Topo I

The camptothecin analogs were quickly developed when their target was recognized as topo I and they were shown to have remarkable anticancer activity against human tumor xenografts propagated in nude mice [102]. Although they are showing activity against a wide range of human malignancies, in general, the response rate of patients with cancer towards these drugs is only in the 20-40% range [103], less than what might have predicted from pre-clinical work in mice [102]. Some human cancers show no response at all [106-110]. There may be several reasons for this. First, as already mentioned, in the presence of human serum albumin, the formation of the inactive carboxylate form of the camptothecins is favored. This does not happen in the presence of mouse serum albumin so that therapy with these drugs in a mouse model system may not be indicative of what occurs in humans.

Second, human cancers may differ markedly in the expression of topo I within a single class of neoplasm. Topotecan has shown to be an effective chemotherapeutic drug for the treatment of

refractory ovarian cancer. The response rate is in the 20-40% range [96,111,112]. Why all patients do not respond is not known. Since it has been clearly shown in the laboratory that sensitivity to topo I targeted drugs is dependent on elevated levels of topo I, perhaps only a subset of ovarian cancers have elevated topo I levels. With the development of an *in situ* immunohistochemical stain to detect topo I on slides prepared from formalin-fixed, paraffin-embedded human tissues, this hypothesis can now be tested. In a study of ovarian cancer, it was recently observed that elevated topo I protein is detected only in about 43% of the tumors [48]. Thus the lack of uniform topo I elevation in all cases of ovarian cancer may play an important role in the sensitivity or resistance of the tumor to topo I specific drug therapy.

Another example of this is in kidney cancer. Topo I targeted drugs have been used in early clinical trials against this malignancy but were not found to be effective [113]. Recent immunohistochemical data [114] and quantitative biochemical measurements [115] provide a possible explanation. It has been observed that levels of topo I in most neoplasms of the kidney are not elevated. The lack of high levels of topo I in this malignancy may help explain the lack of response to topo I targeted drug therapy. If there is no target, there may be no response.

If patient response to topo I targeted chemotherapy depends in part on expression of topo I, which many *in vitro* studies have suggested that it should [87, 90], then it may be possible to identify potentially drug sensitive or drug resistant tumors in a single class of neoplasm by measuring topo I levels.

In addition, the selectivity of the topo I targeted drugs against cancer cells might be explained by the elevations of topo I that have been observed in some human cancers. Human malignancies such as neuroblastomas [116], colorectal cancers [115, 117- 119], myeloma [120], melanoma [121], bladder cancer [122], testicular seminomas [123], brain gliomas [124], head and neck squamous cancers [125], and non-small cell lung cancers [126], have all been shown to contain elevated levels of topo I.

The topo I active drugs are not innocuous because they cause myelosuppression and diarrhea [127], but appear to selectively target malignant cells over normal cells. Although all cells probably contain topo I [128], because the enzyme is not cell cycle specific [129], enzyme levels are probably too low in normal non-proliferating cells to result in extensive toxicity in response to topo I targeted antitumor agents.

The mechanism which results in increased topo I expression in human cancers is not entirely clear, but provocative studies have suggested that it could be related to increased transcription of the gene [115,130], increased topo I gene copy number [131], or decreased degradation of topo I protein [132].

4. Camptothecins are S-phase Specific Drugs

Another important characteristic of a neoplasm that may determine tumor response to topo I targeted drug therapy is the proliferation rate of the neoplasm. It has been clearly documented that the topo I targeted drugs are S-phase specific. The DNA damage they incur must interact with the replication fork in order to be channeled to cell death. In agreement with this, aphidocolin, a DNA polymerase inhibitor, can prevent cell death from camptothecin [133, 134]. Human neoplasms have different characteristics than tumor cells propagated in the laboratory. Although some neoplasms such as high grade non-Hodgkin's lymphomas [135] and small cell lung cancers [136] have a high proportion of proliferating cells, others, like low grade kidney [114] and prostate cancers [137] as well as chronic lymphocytic leukemias (CLL)[138], do not. It might be predicted that human cancers with a large population of non-cycling tumor cells in the G1 phase of the cell cycle, would be resistant to S-phase specific drugs like the camptothecins. This may be true and might help explain why topo I targeted drugs are active against small cell lung cancer [139] and lymphomas [140] (high numbers of cycling tumor cells), but not against prostate

cancer [141], kidney cancer [113], or CLL [107] (low numbers of cycling tumor cells). In fact, this might be another reason the drugs appeared so active in early studies with mice. Human xenografts, propagated in nude mice, have a high proportion of cycling cells (J.Holden, unpublished data). From what is known today, it would be surprising if such tumor xenografts did not respond well to topo I targeted drugs which are S-phase specific.

Evidence has been recently reported to suggest that some camptothecin cytotoxicity may be cell cycle independent [142-144]. This is an important observation, which might be exploited against slow growing neoplasms that do not contain large numbers of cycling cells and would be an important area for future investigation.

5. Apoptosis and Repair of Topo I Targeted Drug Damage

The pathway which leads from topo I drug targeted DNA damage to cell death is not entirely clear. Treatment of cells with these drugs can result in apoptosis [145,146], which might suggest that a functional p53 tumor suppressor gene must be present for the drugs to work. Evidence has been presented which suggests that the p53 protein is necessary to channel DNA damage induced by anticancer drug therapy to cell death via an apoptotic pathway [147]. This might indicate that human cancers which contain mutations in the p53 gene might be resistant to topo I targeted chemotherapy. Because mutations in the p53 gene are common in human neoplasms, this could be another reason why the drugs only target a subpopulation of human malignancies. There may also be p53 independent mechanisms of cell death whose pathways must be intact for anticancer drug therapy against topo I to be successful as well [148].

Several recent studies have been directed towards understanding how damage incurred by topo I targeted drugs might be repaired. A novel enzymatic activity has been reported which has the property of recognizing the covalent complex of topo I and DNA. The enzyme is a phosphodiesterase and will hydrolyze the 3' phosphodiester-tyrosine bond formed between the active site tyrosine on topo I and the broken DNA strand [149]. This topo I-DNA covalent complex is the precise structure, which is expected to be present in cells treated with the camptothecin analogs. The gene for this enzyme has been isolated from yeast [150]. Yeast which contain mutations in the gene are hypersensitive to topo I targeted drugs because they are unable to repair the drug induced DNA damage. This might imply that increased activity of this gene might represent a novel mechanism of resistance to topo I targeted drug therapy.

The gene for this topo I-DNA hydrolase is widely distributed in eukaryotes [150] which suggests that it may play an important role in repairing damage induced by topo I drug therapy in human cells. Its distribution in human malignancies is not known, but if it is present, and its activity can be inhibited, this might make a human tumor exquisitely sensitive to topo I targeted drugs and suggest a novel mechanism for combination chemotherapy.

An additional mechanism of possible resistance to topo I targeted drug toxicity has recently been suggested [151]. Treatment of cells with camptothecin activates NF-kB. The activation of NF-kB results in the expression of genes that have an overall anti-apoptotic effect. This may be a cell's natural resistance to camptothecin induced apoptosis and therefore by inhibiting NF-kB activation a tumor cell might could become extremely sensitive to topo I targeted chemotherapy [152].

As discussed, a variety of mechanisms may play an important role in determining the response of a cancer cell to topo I anticancer drug therapy. Human cancers are more complex than mouse xenografts and in some respects, it is remarkable that the drugs targeting topo I are as active as they are against human malignancies. Clearly topo I targeted drugs have proved their worth in the clinic. Because of their novel mechanism of action, future studies combining them with other active anticancer drugs and radiation [153] might prove beneficial.

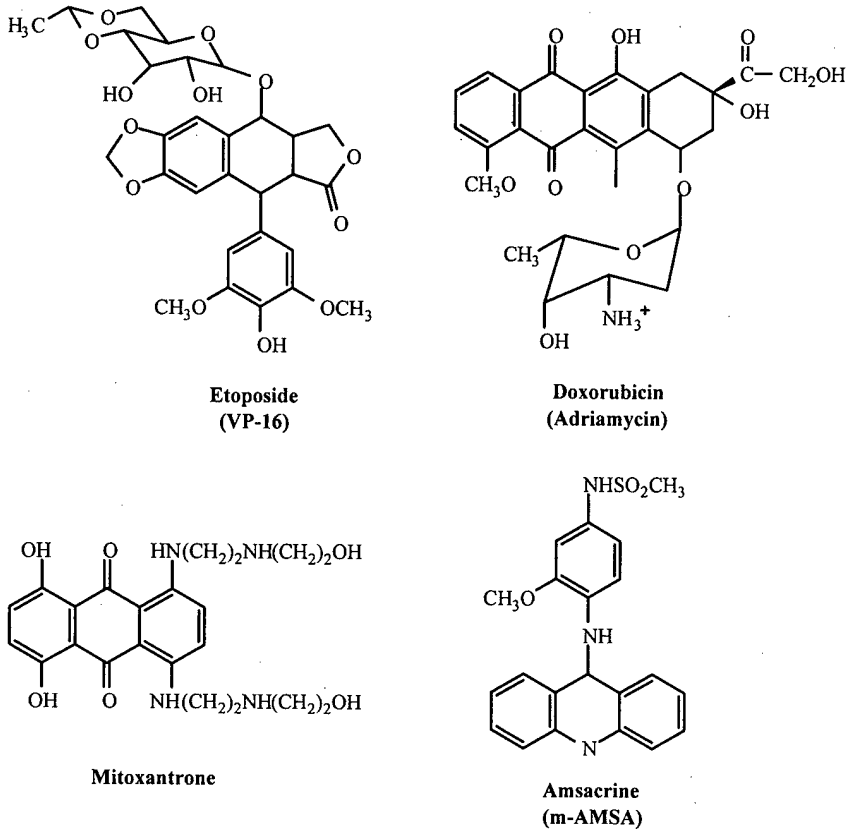


Fig. (5). Molecular structures of topo II targeted anticancer drugs. Some of the most widely used anticancer drugs such as etoposide and doxorubicin have been shown to target topo II.

G. TOPO II TARGETED CHEMOTHERAPY

Anticancer drugs targeting topo II are some of the most widely used chemotherapeutic agents. These drugs include doxorubicin, etoposide, mitoxantrone, and others [6]. In a broad sense, the drugs targeting topo II can be classified as either topoisomerase "poisons" or topoisomerase catalytic inhibitors. As previously discussed, a transient double strand DNA break is characteristic of the topo II enzymatic reaction. The topo II poisons increase the steady state levels of this transient intermediate. They do this either by increasing the rate of DNA cleavage (ellipticine, genistein) or by inhibiting the rate of DNA religation (etoposide, amsascrine, teniposide). In either case, these drugs convert the enzyme into a DNA damaging agent. This is a stochiometric relationship because there is the potential for one DNA double strand break for every drug stabilized topo II enzyme. Thus sensitivity to the topo II poisons is dependent on high levels of enzyme. The more enzyme, the more DNA damage. On the other hand, the topo II catalytic inhibitors act like other types of anticancer drugs and inhibit the activity of the enzyme. Drugs interfering with the catalytic activity of the enzyme deprive the cell of the enzyme's ability to decatenate chromosomal DNA strands prior to mitosis. Cell growth is therefore altered. For these catalytic inhibitors, the reverse sensitivity pattern holds. Cells with low levels of topo II are the most sensitive to the topo II catalytic inhibitors and cells with high topo II levels are most resistant. The topo II poisons resemble the action of the quinolones on *E. coli* gyrase and topo IV, and the catalytic inhibitors resemble the action of the coumarins. The molecular structures of some of the drugs that target topo II are shown in (Fig. **5**).

1. Topoisomerase II Poisons

Drugs such as etoposide, teniposide, doxorubicin, ellipticine, genistein, mitoxantrone and amasacrine are referred to as topoisomerase II poisons. Their primary mechanism of action is to increase

the concentration of the normally short-lived cleaved DNA intermediate that occurs during the topoisomerase II reaction. Theoretically, they could do this either by increasing the forward rate of the cleavage reaction or inhibiting the reverse (religation) reaction rate. In fact, accumulating data indicate that the topoisomerase II poisons can be divided into two classes based on which part of the cleavage reaction they affect. There are drugs that increase the amount of cleaved intermediate by stimulating the forward reaction and there are those that increase the amount of cleaved intermediate by inhibiting religation. The drugs that have clearly been shown to inhibit religation are etoposide, teniposide, and amsacrine [6]. Azotoxin (a molecular hybrid between etoposide and ellipticine) [154], ellipticine [155], and genistein [156] appear to stimulate the forward reaction rate.

For etoposide, which inhibits religation, recent steady state and pre-steady state enzymatic assays have allowed for a precise determination where in the reaction cycle the drug acts [157]. It appears that the drug acts to either inhibit the release of the first ADP from the enzyme or the hydrolysis of the second ATP. The transport of DNA through the G segment occurs prior to the inhibitory effects. Therefore the enzyme, in the presence of etoposide, is able to pass one DNA strand through the break in the G segment, but to catalyze any other strand transfers.

The topoisomerase II poisons are converting the enzyme into a DNA damaging agent. The interesting possibility that this type of topoisomerase mediated DNA damage in the presence of an anticancer drug may actually represent a normal cellular pathway involved in cell death has recently been suggested [158]. Several types of DNA damage such as base mismatches, deaminated cytosines, and the loss of pyrimidine residues, induce topo II DNA cleavage in a manner analogous to the topo II poisons. Perhaps a cell that has suffered from unrepairable DNA damage uses topo II function to cleave DNA at the damaged areas which then cycles the cell to an apoptotic death. The topo II targeted anticancer drugs may simply take advantage of this pathway.

2. Topoisomerase II Catalytic Inhibitors

The DNA topoisomerase II catalytic cycle is complex and consists of six distinct steps [159]. Step 1 is the binding of the enzyme to the DNA; Step 2 is the production of a double strand DNA break prior to strand passage; Step 3 is the strand passage event which occurs in the presence of ATP; Step 4 is the religation of the DNA break occurring after strand passage; Step 5 is the hydrolysis of ATP; and Step 6 is the dissociation and release of the DNA from the topoisomerase. Although several types of drugs have their primary effect on inhibiting topoisomerase enzymatic activity it is clear that there are several places in the topoisomerase enzymatic reaction where these drugs could exert their effect. For example, aclarlubicin inhibits step 1, merbarone and staurosporine inhibit step 2, novobiocin and coumarmycin inhibit step 3 (but primarily in the prokaryotic enzymes) and ICRF-193 inhibits step 5 [160].

As with etoposide, the application of pre-steady state and steady state kinetics have allowed for a precise determination where some of these drugs may act in the enzymatic reaction. Such results have recently been described for the topoisomerase catalytic inhibitor, ICRF-193, a bisdioxopiperazine. These types of drugs are used as cardioprotectants during anthracylcine based chemotherapy [161]. Although they have been shown to inhibit topo II, they have not yet found clinical use as anticancer drugs, but this possibility remains in the future. Initial studies suggested these drugs work as classic topoisomerase catalytic inhibitors. For example, if the effect of these drugs is by inhibiting topoisomerase function and therefore depleting the cell of this vital enzymatic activity, it would be predicted that sensitivity could be overcome by increasing the topoisomerase content in a cell. On the other hand, cells with decreased topoisomerase II would be predicted to be exquisitely sensitive to these agents. These predictions were shown to be true in an experimental yeast system [162]. In addition, it is known that this type of drug traps the topoisomerase molecule in the closed clamp conformation [163]. Recent pre-steady state analysis of the effect of the drug on the enzyme indicates that the drug probably binds to the enzyme after the first ADP has been released

and the second ATP hydrolyzed. The release of the first ADP leaves the enzyme with one empty ATP binding site. The enzyme then becomes locked in the closed clamp formation and unable to undergo repeated turnovers although it can still hydrolyze ATP because of the one empty ATP site [164]. The drug seems to act downstream from etoposide. Although inhibition of enzymatic activity in this manner may be related to the drug's cytotoxicity, the trapped, closed clamp conformation of the enzyme also might interfere with other vital cellular processes resulting in cell death [165]. This could result in cytotoxicity in a mechanism which is distinct from simply inhibiting the catalytic activity of the enzyme or stabilizing the transient cleaved intermediate.

3. Topoisomerase II Expression in Human Tissue and Tumors

The topo II poisons such as etoposide and doxorubicin, are the types of drugs currently used in the treatment of a variety of human cancers. As clearly evident from much laboratory data, sensitivity to this type of drug should be dependent on cellular content of topoisomerase. This leads to the hypothesis that the response of human cancers to topo II therapy may be dependent on, and perhaps predicted by, tumor content of topoisomerase. Unfortunately, it is not easy to determine topoisomerase content on small biopsies of human cancers. Procedures such as Western blot and enzymatic activity measurements are compromised because they are averaging techniques and it is impossible to know how much of the signal observed is due to tumor topoisomerase and how much is due to normal cellular components. Unlike laboratory cell lines, biopsies of human cancers contain, in addition to the tumor cells, abundant normal mesenchymal cells and a wide variety of inflammatory cells.

In order to circumvent this problem as well as to develop an easy clinical test to measure topo II content in human cancers, several *in-situ* immunohistochemical stains for topo II have been developed [126, 166-169]. In these procedures, a slide from a human tumor is first stained for topo II using specific antibodies. Detection of the specific topo antibody is then accomplished by a secondary antibody reaction in which the secondary antibody is coupled to an alkaline phosphatase or horseradish peroxidase system. With the addition of the appropriate chomogen, a colored precipitate occurs wherever the initial topo specific antibody first bound. Topo positive cells appear brown or red when viewed by light microscopy. A strongly positive topo II immunostain of a human lymphoma is shown in (Fig. **6**). Topo II immunohistochemical staining allows for a visual discrimination between the topoisomerase content in the tumor verses the normal cellular components.

Studies with antibodies specific for the alpha and beta isoforms of topo II have indicated that the data derived from the laboratory are applicable to human tissues. Topo II-alpha, identified in the laboratory as a proliferation marker [170] as well as an enzyme essential in meiotic division [171], is only found in human tissues which contain cells undergoing these functions. The enzyme is located in the crypts of the gastrointestinal glands, the basal layer of all squamous and transitional epithelium, the proliferating cells of the ovarian follicle and in the testis where the separation of DNA strands is necessary for proper meiotic division [172]. On the other hand, topo II-beta, identified in the laboratory as an enzyme without cell cycle regulation [72], is uniformly present and easily detectable in all human tissues studied [172]. This latter finding makes one ask the question as to why there should be any therapeutic selectivity of the topo II drugs against cancer. Topo II-beta has been shown to be as sensitive as topo II-alpha to the topo II drugs [173,174] and yet the drugs, although certainly not innocuous, show a therapeutic selectivity against human malignancies. Why should there be any therapeutic selectivity towards a cancer if the beta isoform is present in all human tissues? Perhaps damage mediated through topo II-beta interaction is readily repaired and not channeled to cell death in non-proliferating tissues. It may be that only in a proliferating cancer cell, does topo II-beta drug targeting result in cell killing. Topo II beta is not a cell cycle regulated protein and from what little information is known about topo II-beta levels in human malignancies, it would appear that there is no elevation of the enzyme above normal tissues [126, 172, 175].

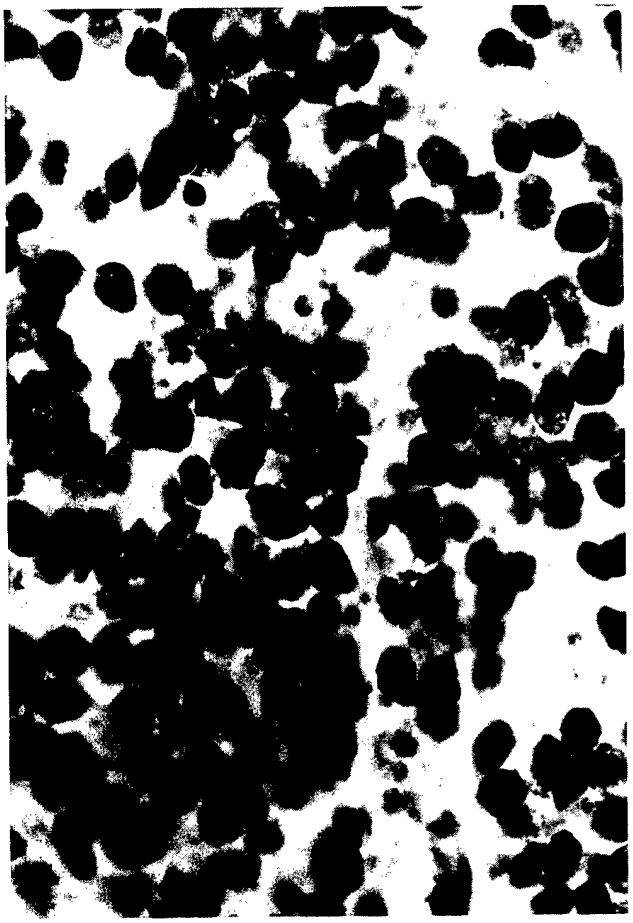


Fig. (6). DNA topoisomerase II immunohistochemical stain. A lymphoblastic lymphoma was immunohistochemically stained with an antibody specific for the alpha isoform of human DNA topoisomerase II. The staining pattern is nuclear and nucleolar. Many positive cells are observed. The percent of positive staining tumor cells is the topo II-alpha index. The lymphoma in the figure has a topo II-alpha index of 92. (original magnification x 400)

However, with the report of drugs which are specific for the beta isoform [176], additional studies concerning the role that this enzyme plays in anticancer drug therapy are clearly needed.

Although studies with topo II-beta in human cancers are limited, this is not true for the alpha isoform. Many investigations have been published evaluating the presence of this isoform in human cancer. Most of these studies are immunohistochemical in nature and have indicated that topo II-alpha identifies the percent of proliferating cells in a neoplasm. The gold standard of proliferation markers in the pathology lab is the MIB1 (Ki-67) antigen. The function of MIB1 is not known but the antigen appears to be present in late G1, S, and the G2 phases of the cell cycle [177]. It is not present during most of G1 and therefore identifies actively proliferating cells. The expression of MIB1 in a tumor can be semi-quantitated by determining a MIB1 index, which represents the percent of positively staining tumor cells. The higher the MIB1 index of a tumor, the greater the number of cells in the proliferative portion of cell cycle. Topo II expression in a tumor can be semi-quantitated immunohistochemically in a similar fashion by determining the topo II-alpha index, which represents the percent of cells staining positively for topo II-alpha [135]. A close correlation between MIB1 indices and topo II-alpha indices have been observed in breast cancer [178], endometrial cancer [179], brain tumors [180, 181], gastric carcinomas [182], cancer of the esophagus [183], colon carcinomas [184], tumors of the adrenal cortex [185], salivary gland carcinomas [186], soft tissue sarcomas [187], uterine cervical cancers [188], ovarian cancer [189], non-Hodgkin's lymphomas [135], and renal cell carcinomas [114]. Obviously, immunohistochemical staining of a tumor for topo II gives information on the number of cycling cells.

Not surprisingly, topo II-alpha expression appears to be highest in malignancies of high clinical aggressiveness. This may reflect the fact that such tumors are composed of an abundant population of proliferating cells. Thus high expression of topo II-alpha has been observed in high grade breast cancers [178, 190], high grade kidney cancers [114], highly aggressive salivary gland tumors [186], high grade squamous cancers of the head and neck [191], high grade non-Hodgkin's lymphomas [135], highly malignant brain tumors [181], aggressive thyroid malignancies [192], and high grade *in situ* breast cancer [193]. These data suggest that the evaluation of topo II-alpha in a human malignancy might be used to help predict prognosis. Several reports suggest that this may be true. Increased levels of topo II-alpha have been shown to be associated with overall poor survival in breast cancer [194,195], small cell lung cancer [196], salivary gland cancers [186], soft tissue sarcomas [187], and surface epithelial neoplasms of the ovary and peritoneum [197].

Apart from patient survival data, the question as to whether expression of topo II in a human cancer predicts response to drugs targeting the enzyme, is more difficult to answer. Clearly laboratory data suggest this may be true. For example, studies indicate that breast cancer cells, resistant to etoposide, can be made sensitive by transfecting them with a recombinant virus which produces active human topo II-alpha [198]. In addition, there is the interesting clinical observation that patients whose breast cancers overexpress the c-erbB-2 oncogene appear more responsive to chemotherapeutic protocols involving topo II targeted drugs than patients whose tumors do not overexpress c-erbB-2 [199]. It is now known that the gene for topo II-alpha is linked to the c-erbB-2 oncogene and simultaneous amplification of both genes may occur in some cases of breast cancer [200]. The increased amount of topo II in such tumor may provide additional drug target and sensitize the tumor to chemotherapy. In addition, small cell lung cancers [136] and testicular seminomas [123], known to be sensitive to the topo II drug etoposide, contain high amounts of topo II while renal cell carcinomas [114] and CLL [138], generally resistant to topo II targeted drugs, do not contain high amounts of enzyme. Of course response to therapy requires more than just a functional drug target. The involvement of genes regulating apoptosis and DNA repair, as well as drug uptake and efflux, may also play important roles. The possibility of determining the drug response of a neoplasm by measuring a single parameter may be too simplistic and may explain why several recent studies showed that topo II expression in advanced breast cancer [201] or in acute myelogenous leukemia [202] did not predict response to chemotherapy. Nonetheless, the accumulating data on topo II expression in human malignancies in conjunction with new data on other molecules involved in anticancer drug response, may help us understand the varied responses of patients with malignant disease to anticancer drug therapy targeted against topo II.

H. CONCLUSIONS

The topoisomerase field has blossomed since the first discovery of this type of enzyme in 1971. The catalytic activities, regulatory properties, gene structures, and crystallographic structures of these molecules are being studied in detail. Because the topoisomerases are targets for a number of important anticancer and antimicrobial drugs, much of the basic research has been translated into the clinical arena. For those who have had the good fortune of working in this area it has been especially satisfying to see insights made at the laboratory bench directly applied in the clinic. Clearly there is more to do and learn. The discovery of new members of the topoisomerase group and the synthesis and discovery of new topoisomerase targeting drugs should provide in the future an even deeper understanding of the role that these fascinating enzymes play in basic cellular biology and in human disease.

I. ACKNOWLEDGEMENTS

Research support from Pharmacia-Upjohn Inc., the Associated Regional and University Pathologists (ARUP) Institute for Clinical and Experimental Pathology, and the Department of Defense (grant # DAMD17-00-1-0665) is gratefully acknowledged.

ABBREVIATIONS

Topo I	=	DNA topoisomerase I
KDa	=	Kilodaltons
Topo III	=	DNA topoisomerase III
Topo IV	=	DNA topoisomerase IV
Topo II	=	DNA topoisomerase II
Kb	=	Kilobase
CLL	=	Chronic lymphocytic leukemia

REFERENCES

[1] Wang, J.C. *J. Mol. Biol.*, **1971**, *55*, 523.

[2] Wang, J.C. *Ann. Rev. Biochem.*, **1996**, *65*, 635.

[3] Pommier, Y. *Biochimie*, **1998**, *80*, 255.

[4] Larsen, A.K.; Gobert, C. *Pathol. Oncol. Res.*, **1999**, *5*, 171.

[5] Bailly, C. *Curr. Med. Chem.*, **2000**, *7*, 39.

[6] Fortune, J.M.; Osheroff, N. *Prog. Nucl. Acid Res.* **2000**, *64*, 221.

[7] Rowe, T.C.; Tewey, K.M.; Liu, L.F. *J. Biol. Chem.* **1984**, *259*, 9177.

[8] Tse-Dinh, Y.C.; Wang, J.C. *J. Mol. Biol.* **1986**, *191*, 321.

[9] Trucksis, M.; Depew, R.E, *Proc. Natl. Acad. Sci. (USA)*, **1981**, *78*, 2164.

[10] Kirkegaard, K.; Pflugfelder, G.; Wang, J.C. *Cold Spring Harb. Symp. Quant. Biol.* **1984**, *49*, 411.

[11] Kirkegaard, K.; Wang, J.C. *J. Mol. Biol.*, **1985**, *185*, 625.

[12] Lima, C.D.; Wang, J.C.; Mondragon, A. *Nature*, **1994**, *367*, 138.

[13] Tse-Dinh, Y. *Biochim. Biophys. Acta*, **1988**, 1400, 19.

[14] Feinberg, H.; Lima, C.D.; Mondragon, A. *Nat. Struct. Biol.* **1999**, *6*, 918.

[15] Pruss, G.J.; Manes, S.H.; Drlica, K. *Cell*, **1982**, *31*, 35.

[16] DiNardo, S.; Voelkel, K.A.; Sternglanz, R. *Cell*, **1982**, *31*, 43.

[17] Masse, E.; Drolet, M. *J. Biol. Chem.*, **1999**, *274*, 16654.

[18] Grishin, N.V. *J. Mol. Biol.* **2000**, *299*, 1165.

[19] Digate, R.J.; Marians, K.J. *J. Biol. Chem.* **1989**, *264*, 17924.

[20] Hiasa, H.; DiGate, R.J.; Marians, K.J. *J. Biol. Chem.* **1994**, *269*, 2093.

[21] Srivenugopal, K.S.; Lockshon, D.; Morris, D.R. *Biochemistry*, **1984**, 23,1899.

[22] Hiasa, H.; Marians, K.J. *J. Biol. Chem.*, **1994**, *269*, 51, 32655.

[23] Wang, H.; DiGate, R.J.; Seeman, N.C. *Proc. Natl. Acad. Sci (USA)*, **1996**, *93*, 9477.

[24] Mondragon, A.; DiGate, R. *Structure*, **1999**, *7*, 1373.

[25] Berger, J.M.; Fass, D.; Wang, J.C.; Harrison, S.C. *Proc. Natl. Acad. Sci. (USA)*, **1998**, *95*, 7876.

[26] Gellert, M.; Mizuuchi, K.; O'Dea, M.H.; Nash, H.A. *Proc. Natl. Acad. Sci (USA)*, **1976**, *73*, 3872.

[27] Levine, C.; Hiasa, H.; Marians, K.J. *Biochem. Biophys. Acta*, **1998**, 1400, 29

[28] Liu, L.F.; Wang, J.C. *Cell* **1978**, *15*, 979.

[29] Lee, M.P.; Sander, M.; Hsieh, T. *J. Biol. Chem.* **1989**, *264*, 21779.

[30] Kato, J.; Nishimura, Y.; Imamura, R.; Niki, H.; Hiraga, S.; Suzuki, H. *Cell*, **1990**. 63, 393.

[31] Peng, H.; Marians, K.J. *J. Biol. Chem.* **1995**, *270*, 25286.

[32] Steck, T.R.; Drlica, K. *Cell*, **1984**, *36*, 1081.

[33] Khodursky, A.B.; Peter, B.J.; Schmid, M.B.; DeRisi, J.; Botstein, D.; Brown, P.O.; Cozzarelli, N.R.; *Proc. Natl. Acad. Sci (USA)*, **2000**, *97*, 9419.

[34] Zechiedrich, E.L; Khodrusky, A.B.; Bachellier, S.; Schneider, R.; Chen, D.; Lilley, D.M.; Cozzarelli, N.R. *J. Biol. Chem.*, **2000**, *275*, 8103.

[35] Berger, J.M. *Biochem. Biophys. Acta*, **1998**, *1400*, 3.

[36] Tsai, F.T.; Singh, O.M.; Skarzynski, T.; Wonacott, A.J.; Weston, S.; Tucker, A.; Pauptit, R.A.; Breeze, A.L.; Poyser, J. P.; O'Brien, R.; Ladbury, J.E.; Wigley, D.B. *Proteins*, **1997**, *28*, 41.

[37] Anderson, V.E.; Zaniewski, R.P.; Kaczmarek, F.S.; Gootz, T.D.; Osheroff, N. *J. Biol. Chem*, **1999**, *50*, 35927.

[38] Anderson, V.E.; Zaniewski, R.P.; Kaczmarek, F.S.; Gootz, T.D.; Osheroff, N. *Biochemistry*, **2000**, *39*, 2726.

[39] Juan, C.; Wang, J.; Liu, A.A.; Whang-Peng, J.; Knutsen, T.; Huebner, K.; Croce, C.M.; Zhang, H.; Wang, J.C.; Liu, L.F. *Proc. Natl. Acad. Sci. (USA)*, **1988**, *85*,8910.

[40] Kunze, N.; Yang, G.; Dolberg, M.; Sundarp, R.; Knippers, R.; Richter, A. *J. Biol. Chem.* **1991**, *268*, 9610.

[41] Yang, G.; Kunze, N.; Baumgartner, B.; Jiang, Z.; Sapp, M.; Knippers, R.; Richter, A. *GENE*, **1990**, *91*, 247.

[42] Pommier, Y.; Pourquier, P.; Fan, Y.; Strumberg, D. *Biochem. Biophys Acta.* **1998**, *1400*, 83.

[43] Gilmour, D.S.; Pflugfelder, G.; Wang, J.C.; Lis, J.T. *Cell*, **1986**, *44*, 401.

[44] Stewart, A.F.; Schutz, G. *Cell*, **1987**, *50*, 1109.

[45] Gilmour, D.S.; Elgin, S.C.R., *Molecul. Cell. Biol.* **1987**, *7*, 141.

[46] Rose, K.M.; Szopa, J.; Han, F.S.; Cheng, Y.C.; Richter, A.; Scheer, U. *Chromosoma*, **1988**, *96*, 411.

[47] Laurincik, J.; Thomsen, P.D.; Hay-Schmidt, A.; Avery, B.; Greve, T.; Ochs, R.L; Hyttel, P. *Biol. Reprod.* **2000**, *62*, 1024.

[48] Holden J.A.; Rahn, M.P.; Jolles, C.J.; Vorobyev, S.V.; Bronstein, I.B. *J. Clin. Pathol. Mol. Pathol.* **1997**, *50*, 247.

[49] Mo, Y.Y.; Wang, P.; Beck, W.T. *Exp. Cell. Res.*, **2000**, *256*, 480.

[50] Zhang, C.X.; Chen, A.D.; Gettel, N.J.; Hsieh, T.S. *Dev. Biol.* **2000**, *222*, 27.

[51] Morham, S.G.; Kluckman, K.D.; Voulomanos, N.; Smithies, O. *Mol. Cell Biol.*, **2000**, *16*, 6804.

[52] Uemura, T.; Morino, K.; Uzawa, S.; Shiozaki, K.; Yanagida, M. *Nucleic Acids Res.*, **1987**, *15*, 9727.

[53] Wallis, J.W.; Chrebet, G.; Brodsky, G.; Rolfe, M.; Rothstein, R. *Cell*, **1989**, *58*, 409.

[54] Kim. R.; Wang, J.C. *J. Biol. Chem.* **1992**, 267,17178.

[55] Wilson, T.M.; Chen, A,D.; Hsieh, T. *J. Biol. Chem.* **2000**, *275*, 1533.

[56] Seki, T.; Seki, M.; Katada, T.; Enomoto, T. *Biochem. Biophys. Acta*, **1998**, *1396*, 12.

[57] Seki, T.; Seki, M.; Onodera, R.; Katada, T.; Enomoto, T. *J. Biol. Chem.* **1998**, *273*, 28553.

[58] Hanai, R.; Caron, P.R.; Wang, J.C. *Proc. Natl. Acad. Sci. (USA)*, **1996**, *93*, 3653.

[59] Ng, S.W.; Liu, Y.; Hasselblatt, K.T.; Mok, S.C.; Berkowitz, R.S. *Nucleic Acids Res.* **1999**, *27*, 993.

[60] Kawasaki, K.; Minoshima, S.; Nakato, E.; Shibuya, K.; Shintani, A.; Schmeits, J.L.; Wang, J.; Shimizu, N.J. *Genome Res.* **1997**, *7*, 250.

[61] Li, W.; Wang, J.C. *Proc. Natl. Acad. Sci. (USA)*, **1998**, *95*, 1010.

[62] Gangloff, S.; de Massey, B.; Arthur, L.; Rothstein, R.; Fabre, F. *EMBO J*, **1999**, *18*, 1701.

[63] Goulaouic, H.; Roulon, T.; Flamand, O.; Grondard, L.; Lavelle, F.; Riou, F. *Nucleic Acids Res.* **1999**, *27*, 2443.

[64] Kim, Y.C.; Lee, J.; Koo, H.S. *Nucleic Acids Res.* **2000**, *28*, 2012.

[65] Hotoda, N.; Hanai, R. *J. Biochem.* **2000**, *127*, 1109.

[66] Harmon, F.G.; DiGate, R.J.; Kowalczykowski, S.C. *Molecul. Cell*, **1999**, *3*, 611.

[67] Shimamoto, A.; Nishikawa, K.; Kitao, S.; Furuichi, Y. *Nucleic Acids Res.* **2000**, *28*, 1647.

[68] Bennett, R.J.; Noirot-Gros, M. F.; Wang, J.C. *J. Biol. Chem.* **2000**, *275*, 26898.

[69] Wu, L.; Davies, S.L.; North, P.S.; Goulaouic, H.; Riou, J.; Turley, H.; Gatter, K.C.; Hickson, I.D. *J. Biol. Chem.* **2000**, *275*, 9636.

[70] Johnson, F.B.; Lombard, D.B.; Neff, N.F.; Mastrangelo, M.A.; Dewolf, W.; Ellis, N.A.; Marciniak, R.A.; Yin, Y.; Jaenisch, R.; Guarente, L. *Cancer Res.* **2000**, *60*, 1162.

[71] Lodge, A.J.; Anderson, J.J.; Ng, S.W.; Fenwick, F.; Steward, M.; Haugk, B.; Horne, C.H.; Angus, B. *Br. J. Cancer*, **2000**, *83*, 498.

[72] Drake, F.H.; Hofmann, G.A.; Bartus, H. F.; Mattern, M.R.; Crooke, S.T.; Mirabelli, C.K. *Biochemistry*, **1989**, *28*, 8154.

[73] Tsai-Pflugfelder, M.; Liu, L.F.; Liu, A.A.; Tewey, K.M.; Whang-Peng, J.; Knutsen, T.; Huebner, K.; Croce, C.M.; Wang, J.C. *Proc. Natl. Acad. Sci. (USA)*, **1988**, *85*, 7171.

[74] Tan, K.B.; Dorman, T.E.; Falls, K. M.; Chung, T.D.; Mirabelli, C.K.; Crooke, S.T.; Mao, J. *Cancer Res.* **1992**, *52*, 231.

[75] Sng, J.H.; Heaton, V.J.; Bell, M.; Maini, P.; Austin, C.A.; Fisher, L.M. *Biochim. Biophys. Acta*, **1999**, 1444, 395.

[76] Rybenkov, V.V.; Ullsperger, C.; Vologodskii, A.V.; Cozzarelli, N.R. *Science*, **1997**, *277*, 690.

[77] DiNardo, S.; Voelkel, K.; Sternglanz, R. *Proc. Natl. Acad. Sci. (USA)*, **1984**, *81*, 2616.

[78] Earnshaw, W.C.; Halligan, B.; Cooke, C.A.; Heck, M.M.; Liu, L.F. *J. Cell Sci.* **1985**, 100,1706.

[79] Yang, X.; Li, W.; Prescott, E.D.; Burden, S.J.; Wang, J.C. *Science*, **2000**, *287*, 131.

[80] Wang, Y.; Knudsen, B.R.; Bjergback, L.; Westergaard, O.; Andersen, A.H. *J. Biol. Chem.* **1999**, *274*, 22839.

[81] Baird, C.L.; Harkins, T.T.; Morris, S.K.; Lindsley, J.E. *Proc. Natl. Acad Sci. (USA)*, **1999**, 24,13685.

[82] Lynn, R.; Giaever, G.; Swanberg, S.L.; Wang, J.C. *Science*, **1986**, *233*, 647.

[83] Mirski, S.E.; Gerlach, J.H.; Cummings, H.J.; Zirngibl, R.; Greer, P.A.; Cole, S.P. *Exp. Cell Res.* **1997**, *237*, 452.

[84] Cowell, I.G.; Willmore, E.; Chalton, D.; Marsh, K.L.; Jazrawi, E.; Fisher, L.M.; Austin, C.A. *Exp. Cell Res.* **1998**, *243*, 232.

[85] Wall, M.E.; Wani, M. *Ann. N. Y. Acad. Sci.*, **1996**, *803*, 1.

[86] Hsiang, Y.H.; Hertzberg, R.; Hecht, S.; Liu, L.F. J. *Biol. Chem.* **1985**, *260*, 14873.

[87] Bjornsti, M.A.; Benedetti, P.; Viglianti, G.A.; Wang, J.C. *Cancer Res.* **1989**, *49*, 6318.

[88] Benedetti, P.; Fiorani, P.; Capuani, L.; Wang, J.C. *Cancer Res.* **1993**, *53*, 4343.

[89] Pommier, Y.; Pourquier, P.; Urasaki, Y.; Wu, J.; Laco, G. *Drug Resistance Update.* **1999**, *2*, 307.

[90] Hann, C.; Evans, D.L.; Fertala, J.; Benedetti, P.; Bjornsti, M.A.; Hall, D. J. *J. Biol. Chem.*, **1998**, *273*, 8425.

[91] Nitiss, J.L; Rose, A.; Sykes, K.C.; Harris, J.; Zhou, J., Ann. N. Y. *Acad. Sci.*, **1996**, *803*, 32.

[92] Stewart, L.; Redinbo, M.R.; Qiu, X.; Hol, W.G.J.; Champoux, J.J. *Science*, **1998**, *279*, 1534.

[93] Fan, Y.; Weinstein, J. N.; Kohn, K. W.; Shi, L. M.; Pommier, Y. *J. Med. Chem.* **1998**, *41*, 2216.

[94] Nitiss, J.L; Wang, J.C. *Mol. Pharmacol.* **1996**, *50*, 1095.

[95] Strumberg, D.; Pilon, A.A.; Smith, M.; Hickey, R.; Malkas, L.; Pommier, Y. *Mol. Cell. Biol.* **2000**, *20*, 3977.

[96] McGuire, W.P.; Blessing, J.A.; Bookman, M.A.; Lentz, S.S.; Dunton, C.J. *J. Clin. Oncol.*, **2000**, *18*, 1062.

[97] Cunningham, D.; Pyrhonen, S.; James, R.D.; Punt, C.J.; Hickish, T.G.; Heikkila, R.; Johannesen, T.B.; Starkhammar, H.; Topham, C.A.; Awad, L.; Jacques, C.; Herait, P. *Lancet*, **1998**, *352*, 1413.

[98] Rougier, P.; Van Cutsem, E.; Bajetta, E.; Niederle, N.; Possinger, K.; Labianca, R.; Navarro, M.; Morant, R.; Bleiberg, H.; Wils, J.; Awad, L.; Herait, P.; Jacques, C. *Lancet*, **1998**, *352*, 1407.

[99] Saltz, L.B.; Cox, J.V.; Blanke, C.; Rosen, L.S.; Fehrenbacher, L.; Moore, M.J.; Maroun, J.A.; Ackland, S.P.; Locker, P.K.; Pirotta, N.; Elfring, G.L.; Miller, L.L. *N. Engl. J. Med.* **2000**, *343*, 905.

[100] Burke, T.B. *Ann. N. Y. Acad. Sci.*, **1996**, *803*, 29.

[101] Mi. Z.; Burke, T.G. *Biochemistry*, **1995**, *34*, 13722.

[102] Giovanella, B.C.; Hinz, H.R.; Kozielski, A.J.; Stehlin, J.S.; Silber, R.; Potmesil, M. *Cancer Res.* **1991**, *51*, 3052.

[103] Takimoto, CH.; Wright, J.; Arbuck, S.G. *Biochem. Biophys, Acta*, **1998**, *1400*, 107.

[104] Holden, J.A.; Wall, M.E.; Wani, M.C.; Manikumar, G. *Arch.Biochem. Biophys.* **1999**, *370*, 66.

[105] Wadkins, R.M.; Potter, P.M.; Vladu, B.; Marty, J.; Mangold, G.; Weitman, S.; Manikumar, G.; Wani, M.C.; Wall, M.E.; Von Hoff, D.D. *Cancer Res.* **1999**, *59*, 3424.

[106] O'Brien, S.; Kantarjian, H.; Ellis, A.; Zwelling, L.; Estey, E.; Keating, M. *Cancer*, **1995**, *75*, 1104.

[107] Blaney, S.M.; Phillips, P.C.; Packer, R.J.; Heideman, R.L.; Berg, S.L.; Adamson, P.C.; Allen, J.C.; Sallan, S.E.; Jakacki, R.I.; Lange, B.J.; Reaman, G.H.; Horowitz, M.E.; Poplack, D.G.; Balis, F.M. *Cancer*, **1996**, *78*, 527.

[108] Irvin, W.P.; Price, F.V.; Bailey, H.; Gelder, M.; Rosenbluth, R.; Durivage, H.J.; Potkul, R.K. *Cancer*, **1998**, *82*, 328.

[109] Asbury, R.F.; Lipsitz, S.; Graham, D.; Falkson, C.I.; Baez, L.; Benson, A.B. Am. *J. Clin. Oncol.*, **2000**, *23*, 45.

[110] Macdonald, J.S.; Jacobson, J.L.; Ketchel, S.J.; Weiss, G.; Taylor, S.; Mills, G.; Kuebler, J.P.; Rivkin, S.; Conrad, M. *Invest. New Drugs*, **2000**, *18*, 199.

[111] Hochster, H.; Wadler, S.; Runowicz, C.; Liebes, L.; Cohen, H.; Wallach, R.; Sorich, J.; Taubes, B.; Speyer, J. *J. Clin. Oncol.*, **1999**, *17*, 2553.

[112] ten Bokkel Huinink W.; Gore, M.; Carmichael, J.; Gordon, A.; Malfetano, J.; Hudson, I.; Broom, C.; Scarabelli, C.; Davidson, N.; Spanczynski, M.; Bolis, G.; Malmstrom, H.; Coleman, R.; Fields, S.; Heron, J. *J. Clin. Oncol.* **1997**, *15*, 2183.

[113] Law, T.M.; Ilson, D.H.; Motzer, R.J. *Invest. New Drugs*, **1994**, *12*, 143.

[114] Gupta, D.; Bronstein, I.B.; Holden, J.A. *Hum. Pathol.* **2000**, *31*, 214.

[115] Husain, I.; Mohler, J.L.; Seigler, H.F.; Besterman, J.M. *Cancer Res.* **1994**, *54*, 539.

[116] Vassal, G.; Pondarre, C.; Cappilli, C.; Terrier-Lacombe, M.J.; Boland, I.; Morizet, J.; Benard, J.; Vanuai, A.M.; Ardouin, P.; Hartmann, O.; Gouyett, A. *Eur. J. Cancer*, **1997**, 2011.

[117] Mcleod, H.L.; Oates, D.F.; Symonds, R.P.; Prakash, D.; van der Zee, A.G.; Kaye, S.B.; Brown, R.; Keith, W.N. *Int. J. Cancer*, **1994**, *59*, 607.

[118] Giovanella, B.C.; Stehlin, J.S.; Wall, M.E.; Wani, M.C.; Nicholas, A.W.; Liu, L.F.; Silber, R.; Potmesil, M. *Science*, **1989**, *246*, 1048.

[119] Staley, B.E.; Samowitz, W.S.; Bronstein, I.B.; Holden, J.A. *Mod. Pathol.* **1999**, *12*, 356.

[120] Ishikawa, H.; Kawano, N.M.; Okada, K.; Tanaka, H.; Tanabe, O.; Sakai, A.; Asaoku, H.; Iwata, K.; Nobuyoshi, M.; Kuramoto, A. *Br. J. Haematol*, **1993**, *83*, 68.

[121] Lynch, B.J.; Komaromy-Hiller, G.; Bronstein, I.B.; Holden, J.A. *Hum. Pathol.* **1998**, *29*, 1240.

[122] Monnin, K.A.; Bronstein, I.B.; Gaffney, D.K.; Holden, J.A. *Hum. Pathol.* **1999**, *30*, 384.

[123] Coleman, L.W.; Perkins, S.L.; Bronstein, I.B.; Holden, J.A. *Hum. Pathol.* **2000**, *31*, 728.

[124] Matsumoto, Y.; Fujiwara, T.; Honjo, Y.; Sasaoka, N.; Tsuchida, T.; Nagao, S. *J. Surg. Oncol.*, **1993**, *53*, 97.

[125] Masin, J.S; Berger, S.J.; Setrakian, S.; Stepnick, D.W.; Berger, N.A. *Laryngoscope*, **1995**, *105*, 1191.

[126] Giaccone, G.; van Ark-Otte, J.; Scagliotti, G.; Capranico, G.; van der Valk, P.; Rubio, G.; Dalesio, O.; Lopez, R.; Zunino, F.; Walboomers, J.; Pinedo, H.M. *Biochim. Biophys. Acta*, **1995**, 1264, 337.

[127] Ewesuedo, R.B.; Ratain, M.J. *Oncologist*, **1997**, *2*, 359.

[128] Bronstein, I.B.; Vorobyev, S.; Timofeev, A.; Jolles, C.J.; Alder, S.L.; Holden, J.A. *Oncol. Res.* **1996**, *8*, 17.

[129] Heck, M.M.S.; Hittelman, W.; Earnshaw, W.C. *Proc. Natl. Acad. Sci. (USA)*, **1988**, *85*, 1086.

[130] Hwong, C.L.; Chen, C.Y.; Shang, H.F.; Hwang, J. *J. Biol. Chem.* **1993**, *268*, 18982.

[131] Mcleod, H.L.; Keith, W.N. *Br. J. Cancer*, **1996**, *74*, 508.

[132] Chen, H.; Hwong, C.; Wang, C.; Hwang, J. *J. Biol. Chem.* **2000**, *275*, 13109.

[133] Hsiang, Y.; Lihou, M.G.; Liu, L.F. *Cancer Res.* **1989**, *49*, 5077.

[134] Holm, C.; Covey, J.M.; Kerrigan, D.; Pommier, Y. *Cancer Res.* **1989**, *49*, 6365.

[135] Holden, J.A.; Perkins, S.L.; Snow, G.W.; Kjeldsberg, C.R. *Am. J. Clin. Pathol.* **1995**, *104*, 54.

[136] Guinee, D.G.; Holden, J.A.; Benfield, J.R.; Woodward, M.L.; Przygodzki, R.M.; Fishback, N.F.; Koss, M.N.; Travis, W.D. *Cancer*, **1996**, *78*, 729.

[137] Willman, J.H.; Holden, J.A. *The Prostate*, **2000**, *42*, 280.

[138] Potmesil, M.; Hsiang, Y.; Liu, L.F.; Bank, B.; Grossberg, Kirschenbaum S.; Forlenzar, T.J.; Penziner, A.; Kanganis, D.; Knowles, D.; Traganos, F.; Silber, R. *Cancer Res.* **1988**, *48*, 3537.

[139] Masuda, N.; Fukuoka, M.; Kusunoki, Y.; Matsui, K.; Takifuji, N.; Kudoh, S.; Negoro, S.; Nishioka, M.; Nakagawa, K.; Takada, M. *J. Clin. Oncol.* **1992**, *10*, 1225.

[140] Cabanillas, F. *Semin. Hematol*, **1999**, *36*(4 Suppl 8), 11.

[141] Reese, D.M.; Tchekmedyian, S.; Chapman Y.; Prager, D.; Rosen, P.J. *Invest. New Drugs*, **1998**, *16*, 353.

[142] Cohen, D.P.; Adams, D.J.; Flowers, J.L.; Wall, M.E.; Wani, J.C.; Manikumar, G.; Colvin, O.M.; Silber, R. *Leuk. Res.* **1999**, *23*, 1061.

[143] Morris, E.J.; Geller, H.M. *J. Cell Biol.* **1996**, *134*, 757.

[144] Park, D.S.; Morris, E.J.; Stefanis, L.; Troy, C.M.; Shelanski, M.L.; Geller, H.M.; Greene, L.A. *J. Neurosci.* **1998**, *18*, 830.

[145] Zhang, X.W.; Qing, C.; Xu, B. *Anticancer Drugs*, **1999**, *10*, 569.

[146] Pantazis, P.; Chatterjee, D.; Han, Z.; Wyche, J. *Neoplasia*, **1999**, *1*, 231.

[147] Lowe, S.W.; Ruley, H.E.; Jacks, T.; Housman, D.E. *Cell*, **1993**, *74*, 957.

[148] McDonald, A.C.; Brown, R. *Br. J. Cancer*, **1998**, *78*, 745.

[149] Yang, S.; Burgin, A.B.; Huizenga, B.N.; Robertson, C.A.; Yao, K.C.:Nash, H.A. *Proc. Natl. Acad. Sci. (USA)*, **1996**, *93*, 11534.

[150] Pouliot, J.J.; Yao, K.C.; Robertson, C.A.; Nash, H.A. *Science*, **1999**, *286*, 552.

[151] Huang, T.T.; Wuerzberger-Davis, S.M.; Seufzer, B.J.; Shumway, S.D.; Kurama, T.; Boothman, D.A.; Miyamoto, S. *J. Biol. Chem.* **2000**, *275*, 9501.

[152] Wang, C.Y.; Cusack, J.C.; Liu, R.; Baldwin, A.S. *Nat. Med.* **1999**, *5*, 412.

[153] Chen, A.Y.; Choy, H.; Rothenberg, M.L. *Oncology*, **1999**, *13*, (10 suppl 5), 39.

[154] Cline, S.D.; Macdonald, T.L.; Osheroff, N. *Biochemistry*, **1997**, 36,13095.

[155] Froelich-Ammon, S.; Patchan, M.W.; Osheroff, N.; Thompson, R.B. *J. Biol. Chem.* **1995**, *270*, 14998.

[156] van Hille B.; Perrin, D.; Hill, B.T. *Anticancer Drugs*, **1999**, *10*, 551.

[157] Morris, S.K.; Lindsley, J.E. *J. Biol. Chem.* **1999**, *274*, 30690.

[158] Sabourin, M.; Osheroff, N. *Nucleic Acids Res.* **2000**, *28*, 1947.

[159] Burden, D.A.; Osheroff, N. *Biochem. Biophys. Acta.*, **1998**, *1400*, 139.

[160] Fortune, J.M.; Osheroff, N. *J. Biol. Chem.* **1998**, *28*, 17643.

[161] Hasinoff, B.B.; Hellmann, K.; Herman, E.H.; Ferrans, V.J. *Curr. Med. Chem.* **1998**, *5*, 1.

[162] Ishida, R.; Hamatake, M.; Wasserman, R.A.; Nitiss, J.L.; Wang, J.C.; Andoh, T. *Cancer Res.* **1995**, *55*, 2299.

[163] Roca, J.; Ishida, R.; Berger, J.M.; Andoh, T.; Wang, J.C. *Proc. Natl. Acad. Sci. (USA)*, **1994**, *91*, 1781.

[164] Morris, S.K.; Baird, C.L.; Lindsley, J.E. *J. Biol. Chem.* **2000**, *275*, 2613.

[165] Jensen, L.H.; Nitiss, K.C.; Rose, A.; Dong, J.; Zhou, J.; Hu, T.; Osheroff, N.; Jensen, P.B.; Schested, M.; Nitiss, J.L. *J. Biol. Chem.* **2000**, *275*, 2137.

[166] Holden, J.A.; Snow, G.W.; Perkins, S.L.; Kjeldsberg, C.R. *Mod. Pathol.* **1994**, *7*, 829.

[167] Jarvinen, T.; Kononen, J.; Pelto-Huikko, M.; Isola, J. *Am. J. Pathol.* **1996**, *148*, 2073.

[168] Kellner, U, Heidebrecht, H.; Rudoloph, P.; Biersack H.; Buck, F.; Dakowski, T.; Wacker, H.H.; Domanowski, M.; Seidel, A.; Westergaard, O.; Parwaresch, R. *J. Histochem. Cytochem.* **1997**, *45*, 251.

[169] Hellemans, P.; van Dam, P.A.; Geyskens, M.; van Oosterom, A.T.; Buytaert, P.; Van Marck, E. *J. Clin. Pathol.* **1995**, *48*, 147.

[170] Heck, M.; Earnshaw, W.C. *J. Cell Biol.* **1986**, *103*, 2569.

[171] Hartsuiker, E.; Bahler, J.; Kohli, *J. Mol. Biol. Cell*, **1998**, *9*, 2739.

[172] Bauman, M.E.; Holden, J.A.; Brown, K.A.; Harker, W.G.; Perkins, S.L. *Mod. Pathol*, **1997**, *10*, 168.

[173] Austin, C.A.; Marsh, K.L.; Wasserman, R.A.; Willmore, E.; Sayer, P.J.; Wang, J.C.; Fisher, L.M. *J. Biol. Chem.* **1995**, *270*, 15739.

[174] Cornarotti, M.; Tinelli, S.; Willmore, E.; Zunino, F.; Fisher, L.M.; Austin, C.A.; Capranico, G. *Mol. Pharmacol.* **1996**, *50*, 1463.

[175] Mirski, S.E.; Voskoglou-Nomikos, T.; Young, L.C.; Deeley, L.C.; Campling, B.G.; Gerlach, J.H.; Cole, S.P. *Lab. Invest.* **2000**, *80*, 787.

[176] Goa, H.; Huang, K.C.; Yamasaki, E.F.; Chan, K.K.; Chohan, L.; Snapka, R.M. *Proc. Natl. Acad. Sci. (USA)*, **1999**, *96*, 12168.

[177] Endl, E.; Gerdes, J. *Exp. Cell Res.* **2000**, *257*, 231.

[178] Lynch, B.J.; Guinee, D.G.; Holden, J.A. *Hum. Pathol.* **1997**, *28*, 1180.

[179] Ito, K.; Sasano, H.; Yabuki, N.; Matsunaga, G.; Sato, S.; Kikuchi, A.; Yajima, A.; Nagura, H. *Mod. Pathol.* **1997**, *10*, 289.

[180] Taniguchi, K.; Wakabayashi, T.; Yoshida, T.; Mizuno, M.; Yoshikawa, K.; Kikuchi, A.; Nakashima, N.; Yoshida, J. *J. Neurosurg.* **1999**, *19*, 477.

[181] Holden, J.A.; Townsend, J.J. *Mod. Pathol.* **1999**, *12*, 1094.

[182] Yabuki, N.; Sasano, H.; Kato, K.; Ohara, S.; Toyota, T.; Nagura, H.; Miyaike, M.; Nozaki, N.; Kikuchi, A. *Am. J. Pathol.* **1996**, *149*, 997.

[183] Ohashi, Y.; Sasano, H.; Yamaki, H.; Shizawa, S.; Kikuchi, A.; Shineha, R.; Akaishi, T.; Satomi, S.; Nagura, H. *Anticancer Res.* **1999**, *19*, 1873.

[184] Fogt, F.; Nikulasson, S.T.; Holden, J.A.; Alder, S.A.; Hallgrimsson, J.; Jessup, M.J.; Lavin, P.T.; Goldman, H. *Mod. Pathol.* **1997**, *10*, 296.

[185] Ilno, K.; Sasano, H.; Yabuki, N.; Oki, Y.; Kikuchi, A.; Yoshimi, T.; Nagura, H. *Mod. Pathol.* **1997**, *10*, 901.

[186] Hirabayashi, S. *J. Oral Pathol.* **1999**, *28*, 131.

[187] Rudolph, P.; Kellner, U.; Chassevent, A.; Collin, F.; Bonichon, F.; Parwaresch, R.; Coindre, J.M. *Am. J. Pathol.* **1997**, *150*, 1997.

[188] Gibbons, D.; Fogt, F.; Kasznica, J. Holden, J.; Nikulasson, S. *Mod. Pathol.* **1997**, *10*, 409.

[189] Martinchick, J.C.; Rahn, M.P.; Jolles, C.J.; Holden, J.A. *Int. J. Oncol.* **1997**, *10*, 1229.

[190] Lynch, B.J.; Holden, J.A.; Buys, S.S.; Neuhausen, S.L.; Gaffney, D.K. *Hum. Pathol.* **1998**, *29*, 1140.

[191] Stathopoulos, G.P.; Kapranos, N.; Manolopoulos, L.; Papadimitriou, C.; Adamopoulos, G. *Anticancer Res.* **2000**, *20*, 177.

[192] Lee, A.; LiVolsi, V.A.; Baloch, Z.W. *Mod. Pathol.* **2000**, *13*, 396.

[193] Rohr, L.R.; Holden, J.A. *Appl Immunohisto. Mol. Morphol.* **1999**, *7*, 14.

[194] Rudolph, P.; MacGrogan, G.; Bonichon, F.; Frahm, S.; de Mascarel, I.; Trojani, M.; Durand, M.; Avril, A.; Coindre, J.M.; Parwaresch, R. *Breast Cancer Res. Treat.* **1999**, *55*, 61.

[195] Depowski, P.L.; Rosenthal, S.L.; Brien, T.P.; Stylos, S.; Johnson, R.L.; Ross, J.S. *Mod. Pathol.* **2000**, *13*, 542.

[196] Dingemans, A.M.; Witlox, M.A.; Stallaert, R.A.; van der Valk, P.; Potmus, P.E.; Giaccone, G. *Clin. Cancer Res.* **1999**, *5*, 2148.

[197] Costa, M.J.; Hansen, C.L.; Holden, J.A.; Guinee, D.G. *Int. J. Gynecol. Pathol.* **2000**, *19*, 248.

[198] Zhou, Z.; Zwelling, L.A.; Kawakami, Y.; An, T.; Kobayashi, K.; Herzog, C.; Kleinerman, E.S. *Cancer Res.* **1999**, *59*, 4618.

[199] Muss, H.B.; Thor, A.D.; Berry, D.; Kute, T.; Liu, E.T.; Koerner, F.; Cirrincione, C.T.; Budman, D.R.; Wood, W.C.; Barcos, M.; Henderson, I.C. *N. Eng. J. Med.* **1994**, *330*, 1260.

[200] Jarvinen, T.; Tanner, M.; Rantanen, V.; Barlund, M.; Borg, A.; Grenman, S.; Isola, J. *Am. J. Pathol.* **2000**, *156*, 839.

[201] Jarvinen, T.A.; Holli, K.; Kuukasjarvi, T.; Isola, J.J. *Br. J. Cancer*, **1998**, *77*, 2267.

[202] Kaufmann, S.H.; Karp, J.E.; Jones, R.J.; Miller, C.B.; Schneider, E.; Zwelling, L.A.; Cowan, K.; Wendel, K.; Burke, P.J. *Blood*, **1994**, *83*, 517.